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14. ABSTRACT

C-peptide is the segment connecting insulin A and B chains. It is generated in pancreatic beta cells as the natural product of pro-insulin cleavage. For a long time, it was considered biologically important only for favoring proinsulin folding within the secretory granules of the beta cells. Consistently with this view, the standard of care for diabetic, and especially T1D patients is solely insulin-replacement therapy; C-peptide is not administrated. However, recent studies have challenged this view. It has been offered increasing evidence that human C-peptide exerts intracellular effects in a variety of cells and could be of real benefit for diabetic patients who suffer from micro-vascular complications. How exactly C-peptide achieves these intracellular effects, however, is still unknown. One major advance in this area would be the identification of the specific C-peptide receptor (CPR) at the level of the cellular membrane and characterization of C-peptide/CPR signaling to effectors upon internalization.

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Annual Report (09/28/2010 – 09/27/2011)
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INTRODUCTION/ORIGINAL STATEMENT OF WORK

Diabetes mellitus is a life-threatening disease that places children (type 1) and adults (type 2) at risk of complications of blindness, kidney damage and heart disease. Diabetes afflicts 16 million Americans, with more than 800,000 new cases diagnosed each year. African, Hispanic, Native and Asian Americans are particularly susceptible to its most severe complications. Costs associated with diabetes are estimated to rich $132 billion/year. Significantly, the number of diabetes cases in the United States military mirrors national rates.

Type 1 diabetes (T1D) patients lack physiological levels of insulin in their bloodstream due to the autoimmune destruction of the insulin producing pancreatic beta cells. Type 2 diabetes (T2D) patients are, instead, afflicted by an heterogeneous set of sub-syndromes characterized by peripheral insulin resistance with or without production insufficiency. Both T1D and T2D patients are at increased risk for damages of both micro- and macro-vascular tissues, which eventually bring to the well known, tragic, diabetic complications.

C-peptide is the segment connecting insulin A and B chains. It is generated in pancreatic beta cells as the natural product of pro-insulin cleavage. For a long time, it was considered biologically important only for favoring pro-insulin folding within the secretory granules of the beta cells. Consistently with this view, the standard of care for diabetic, and especially T1D patients is solely insulin-replacement therapy; C-peptide is not administrated. However, recent studies have challenged this view. It has been offered increasing evidence that human C-peptide exerts intracellular effects in a variety of cells and could be of real benefit for diabetic patients who suffer from micro-vascular complications. How exactly C-peptide achieves these intracellular effects, however, is still unknown.

In preliminary results, we have demonstrated that C-peptide reduces secretion of inflammatory cytokines from endothelial cells in a model of hyperglycemia-induced vascular injury by reducing activation of the nuclear factor (NF)-κB pathway (1). We found a similar anti-inflammatory activity of C-peptide in vascular smooth muscle cells (2). For the full-length, native, C-peptide, we found that, upon internalization from the cell surface, C-peptide quickly traffics to early endosomes and later proceeds to lysosomes for degradation (3). Trafficking of C-peptide to early endosomes is likely to account for its anti-inflammatory effects in vascular endothelial and smooth muscle cells. Based on these findings, it is hypothesized that C-peptide first binds to its cell surface receptor, then the complex internalizes and signals to effector pathways via endosomes (4). One major advance in this area would be the identification of the specific C-peptide receptor (CPR) at the level of the cellular membrane and characterization of C-peptide/CPR signaling to effectors upon internalization (5). In this project, we will set a number of experiments specifically designed to isolate the CPR and characterize its intracellular signaling activity, with the following specific aims:

1. **To isolate the C-Peptide Receptor (CPR).** We have designed and synthesized a set of biotinylated C-peptides including wild type and two mutants previously shown to not bind cellular surface membrane. These peptides will be allowed to internalize into endothelial and smooth muscle cells. Endosomes containing the biotinylated C-peptides will be isolated by cell fractionation, solubilized, and the C-peptide/CPR complexes isolated. Proteins of the wild type but not the mutant C-peptide/ receptor complexes will be sequenced.

2. To identify endosomes as the subcellular site of C-peptide/CPR signaling. Endosomes are likely candidate sites for intracellular signaling by the C-peptide/CPR complex. In this aim, we will block C-peptide internalization at different stages, by using pertussis toxin and endosomal Rab5 trafficking mutants, to determine at which station along its endocytic route C-peptide activates its intracellular signaling pathway.

3. To investigate the anti-inflammatory effect of C-peptide on high glucose-induced vascular dysfunction *in vivo*. We have definite proof that C-peptide displays a powerful anti-inflammatory effect on endothelial *cells in vitro*. It is important to investigate whether this anti-inflammatory activity of C-

peptide is also observed *in vivo*. To this aim, we will inject C-peptide in a mouse model of diabetes-induced vascular disease and study the effect on adhesion molecule expression and macrophage accumulation particularly in the aortic segment.

Our first quarterly scientific progress report for the initial year of our project (09/28/10 – 12/27/10) described the following:

High glucose is toxic to endothelial cells (EC) through generation of reactive oxygen species (ROS).

In the vascular endothelium, the prolonged exposure to high blood sugar (hyperglycemia), in conjunction with other inflammatory insults, causes massive changes in the metabolism and physiology of EC leading to a pathological scenario called endothelial dysfunction. One major mechanism underlying hyperglycemia-induced endothelial dysfunction is through generation of ROS that are widely recognized to be involved in the development of atherosclerotic process in diabetic patients. The excessive production of ROS caused by hyperglycemia leads to severe change in EC proliferation and adhesion property, contributing to accelerate the activation of apoptotic process in the endothelium (4).

Hyperglycemia-induced generation of ROS in endothelial cells occurs mainly through a NAD(P)H oxidase-dependent mechanism. NAD(P)H oxidase is an enzymatic complex made up by several subunits located in the cytoplasm and plasma membrane. Specifically, NAD(P)H oxidase is composed by 2 membrane subunits ($p22^{phox}$ and $gp91^{phox}$) and 4 cytosolic subunits ($p40^{phox}$, $p47^{phox}$, $p67^{phox}$ and Rac-1). In a scenario of hyperglycemia, NAD(P)H oxidase is stimulated and the cytosolic subunits translocate from the cytosol to the membrane to induce ROS production. Elevated production of ROS in the endothelium leads to activation of transcription factors, such as nuclear factor (NF)-κB, which promote transcription of genes involved in inflammatory responses, such as upregulating endothelial adhesion molecule expression, secretion of pro-inflammatory cytokines and chemokines from EC.

C-peptide antagonizes high glucose-induced endothelial dysfunction by reducing activation of the nuclear factor(NF)-kB pathway

Previous work from our laboratory demonstrated a protective effect of physiological concentrations of C-peptide on dysfunctional EC (1). Specifically, C-peptide reduces the expression of vascular cell adhesion molecule-1 (VCAM-1) and secretion of the pro-inflammatory chemokines IL-8 and monocyte chemoattractant protein(MCP)-1 from EC. These are important mediators in the process of monocyte adherence to the dysfunctional endothelium leading to atherosclerosis plaque formation. Indeed, C-peptide was also able to significantly reduce the adherence of monocytes to EC exposed to high glucose *in vitro,* as compared to EC exposed to high glucose alone in the absence of C-peptide. Heat inactivated C-peptide was inactive. Furthermore, we established the NF-kB pathway as the intracellular target mechanism for the anti-inflammatory activity of C-peptide on EC under the damaging influence of high glucose. However, to date it is not known which NF-kB dependent upstream signaling events are affected by C-peptide in EC, but one likely target is represented by ROS generation (1).

Aim

ROS generation represents an early step in high glucose-induced endothelial dysfunction. We have explored the ability of C-peptide to interfere with the generation of ROS in EC exposed to hyperglycemia. As *in vitro* model system, Human Aortic Endothelial Cells (HAEC) were exposed to high glucose (25mM) - in order to mimic the diabetic condition – in the presence or absence of C-peptide, and ROS generation will be evaluated over time.

We also investigated whether C-peptide affects the activity of the enzymatic complex called NAD(P)H oxidase, which is one of the major sources of ROS production in EC. To this aim, we studied the effect of C-peptide on the translocation of the NAD(P)H oxidase cytoplasmic subunit Rac-1 to the plasma membrane. Rac-1 is a small GTPase which is critical for activation of NAD(P)H oxidase complex.

Experiment 1. Determination of ROS generation in HAEC using Flow Cytometry. We evaluated the effect of C-peptide (10nM) on the generation of ROS after overnight incubation in high glucose-activated HAEC. ROS production was assessed using a fluorescent dye called carboxy-DCFDA, which is a cell-permeant indicator for ROS generation. The dye is non-fluorescent until its acetate group is removed by intracellular esterase and oxidation occurs within the cell. Oxidation of the probe was detected by monitoring the increase in fluorescence with Flow Cytometry. The higher the fluorescence detected by flow cytometry, the higher is the level of oxidation in the system. Given the rapidity of ROS turnover, ROS measurements were performed every hour for five hours (0-5hours). C-peptide with randomized sequence (scrambled C-peptide) was used as negative control. Scrambled C-peptide contains the same amino acid sequence of full-length C-peptide but in random order. This experiment was performed at least 3 times.

As shown in **Figure 1**, exposure of HAEC to high glucose (HG) (red) triggered ROS production compared to normal glucose treatment (blue). ROS production by HG reached a peak after 3 hours and then declined. Addition of C-peptide to HG (yellow) significantly decreased ROS production compared to HG alone ($p<0.01$) both at 3 and 4 hours, while scrambled C-peptide was not effective (green). **Figure 1** also shows examples of flow cytometry histograms showing increased fluorescence after HG compared to normal glucose, and decreased fluorescence after C-peptide addition.

Experiment 2. Determination of IL-8 secretion in supernatant from high glucose-exposed HAEC. Cell culture supernatant was collected from HAEC during determination of ROS (as above) and tested for IL-8 content. IL-8 is an inflammatory cytokine secreted by EC during inflammation. As shown in **Figure 2**, HG significantly stimulated IL-8 production compared to normal glucose (NG) ($p<0.05$). Addition of C-peptide to HG, significantly reduced IL-8 production as compared to HG ($p<0.05$) to levels found in NG, while scrambled C-peptide had no effect on IL-8 secretion.

Figure 1

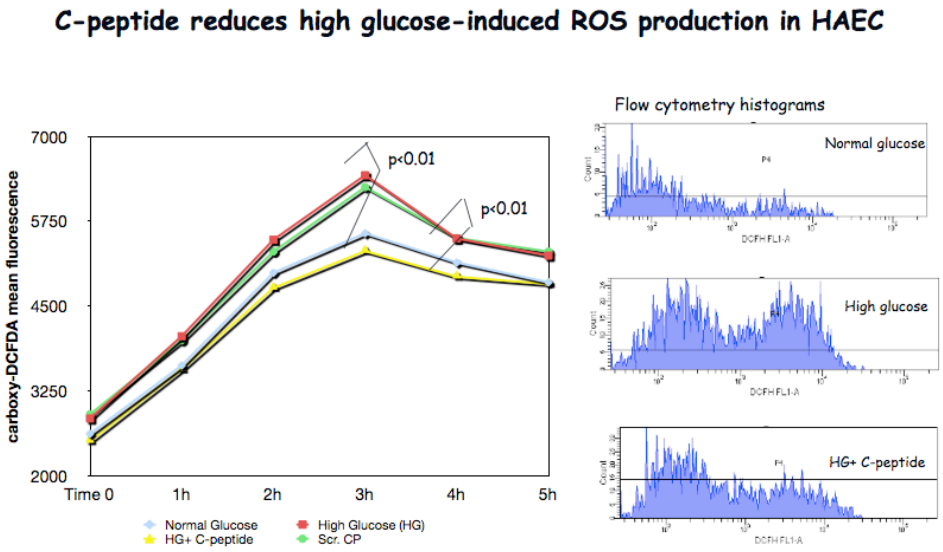
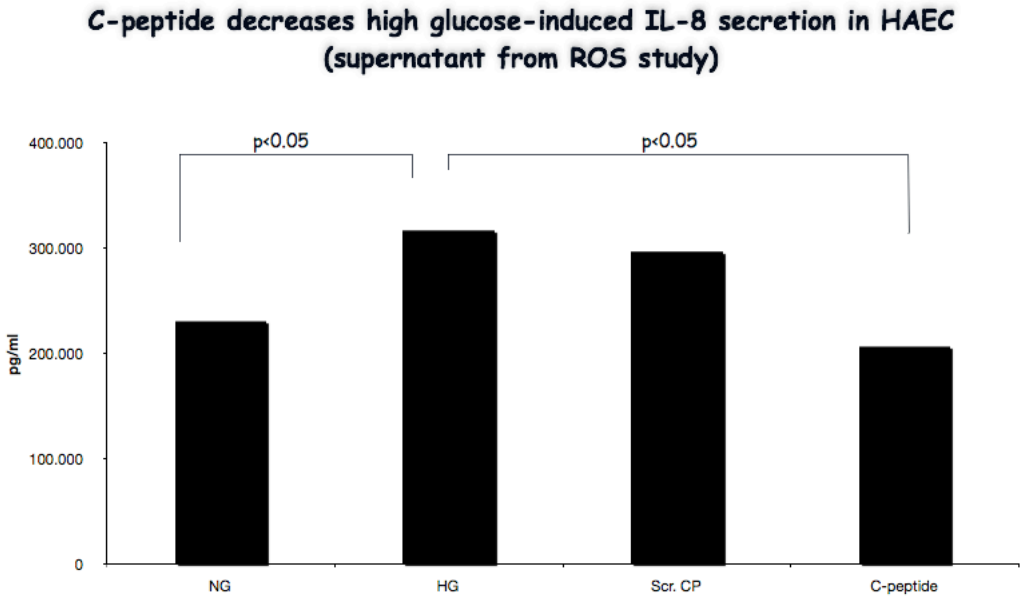


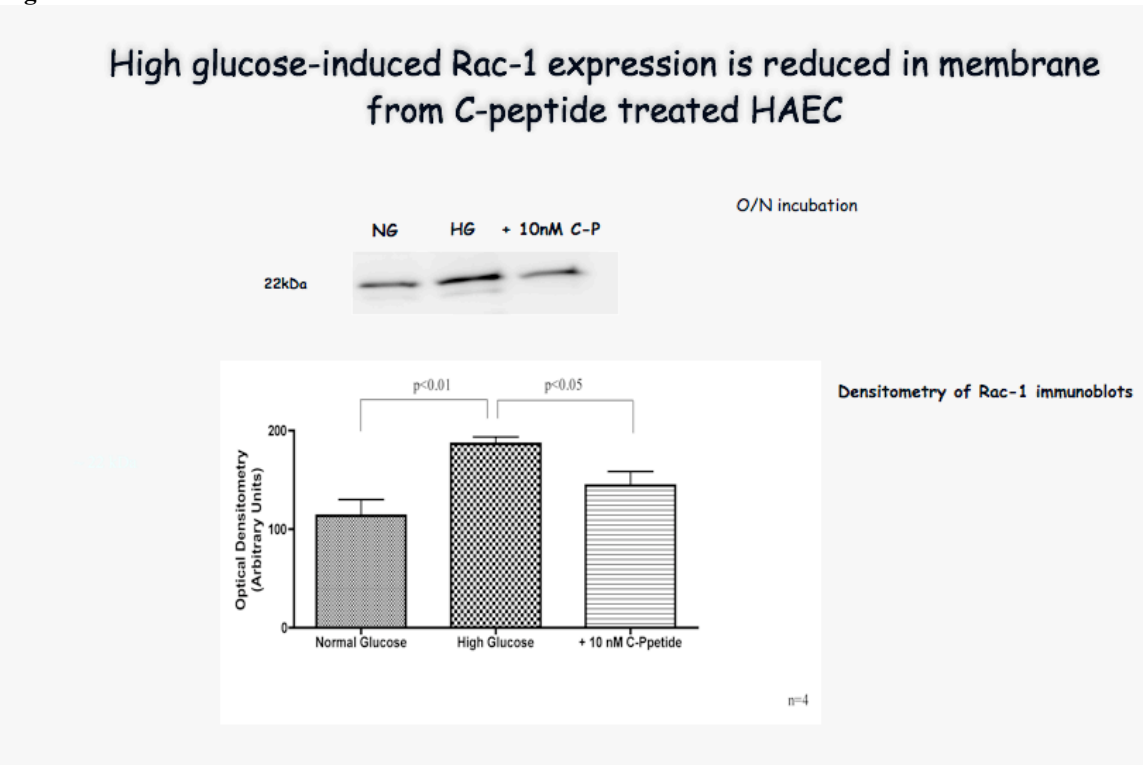
Figure 2



Experiment 3. Changes in expression of the NAD(P)H oxidase subunit Rac-1 in HAEC exposed to high glucose. The expression of Rac-1, in EC was investigated using Western blotting. HAEC were serum starved overnight, and successively treated with high glucose, in presence or absence of 10 nM C-peptide for 30 minutes. Scrambled C-peptide was be used as negative control. For the Western blotting, samples underwent protein isolation, a procedure that allows fractionation of proteins from plasma membrane, cytosol, nuclear and cytoskeleton compartments. Protein concentration was determined by the Bicinchoninic Acid (BCA) protein assay protocol (Pierce, Rockford,IL) using bovine serum albumin as standard. For detection of Rac-1 subunit, 30 μg of cytoplasmic and membrane proteins were run using 4-20% SDS polyacrylamide gels. Proteins separated by SDS/PAGE, were transferred to nitrocellulose membrane and incubated with human anti-Rac-1 (1:500; Santa Cruz Biotechnology) or anti-β-actin (1:10,000; Sigma) antibody at 4°C overnight. The following day, membranes were washed and incubated with a 1:5000 dilution of either rabbit or mouse Ig horseradish peroxide-conjugated antibody (Jackson Laboratories). Bound antibodies were detected by enhanced chemiluminescence (ECL, Amersham). To quantify and compare levels of proteins, the density of each band was be measured by densitometry. Densitometry analysis of the band will be performed with UN-SCAN-IT gel Software (Silk Scientific).

As shown in **Figure 3**, Rac-1 expression in plasma membrane from high glucose(HG)-exposed HAEC increased as compared to cells exposed to normal glucose (NG) ($p<0.01$). C-peptide addition to HG, significantly reduced Rac-1 expression on plasma membrane to levels detected under NG, suggesting decreased activity of the NAD(P)H oxidase enzyme.

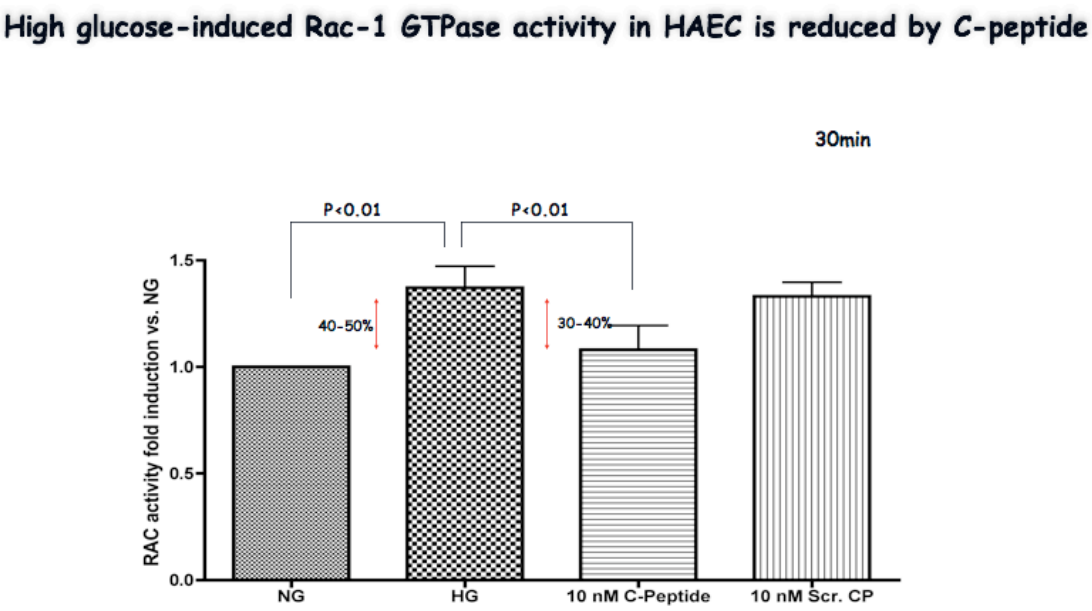
Figure 3



Experiment 4. Measurement of Rac-1 activity in HAEC. Rac-1 belongs to the Rho family of small GTPases which consists of at least 20 members, the most extensively characterized of which are the RhoA, Rac1 and Cdc42 proteins. In common with other small GTPases, the Rac-1 proteins act as molecular switches that transmit cellular signals through downstream effector proteins by alternating between active GTP-bound and inactive GDP-bound states. The Rho family mediates a wide range of cellular responses, including cytoskeletal reorganization, regulation of transcription, membrane trafficking and apoptosis. For this assay, HAEC were serum starved overnight, and successively treated with high glucose (HG), in presence or absence of 10nM C-peptide for 30 minutes. After treatment, 10 μg of total lysates were transferred into 96-well plate coated with Rac-GTP binding domain (RBD) and incubated at 4 ºC for 1 h. The active GTP-bound form of the Rho-family protein, but not the inactive GDP-bound form, from a biological sample binds to the plate. Bound active Rac-1 protein is then detected by incubation with a specific primary antibody followed by a secondary antibody conjugated to HRP. The signal was developed with chemiluminescence reagents.

As shown in **Figure 4**, Rac-1 GTPase activity significantly increased when HAEC are exposed to HG compared to normal glucose (NG) ($p<0.01$). However, C-peptide reduced Rac-1 GTPase activity of 30-40% compared to HG alone ($p<0.01$), an effect not observed with scrambled C-peptide.

Figure 4



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12. Use additional page(s) to present a brief statement of plans or milestones for the next quarter.

While we are continuing to explore the beneficial effects of C-peptide on the vascular endothelium by studying effects on apoptosis (original specific Aim 3), we already started to perform the experiments aimed at the isolation of the C-Peptide Receptor (CPR) as presented in our original Aims 1 and 2.

We are working toward this goal by performing the following experiments:

1. We are planning to use a set of biotinylated C-peptides, including a wild-type and a randomized version of C-peptide, in an effort to follow the physiological internalization of C-peptide upon its binding to the putative CPR. These experiments are based on our preliminary data showing that C-peptide rapidly internalizes in endothelial and vascular smooth muscle cells and localizes to early endosomes (3). The use of biotinylated peptides in pull-down experiments will allow identification of the specific interacting proteins that will then be sequenced. We expect that the proteins that are pulled-down by the

biotinylated wild-type C-peptide be different that the ones from the scrambled C-peptide probe. We are currently improving the procedure to obtain purified endosomes from our cells *in vitro*.

2. It has been suggested that the CPR is a G-protein-coupled receptor from studies in which pertussis toxin inhibits the binding to cell membrane and some intracellular signaling (4,5). Based on these preliminary results, we are planning to knock down gene expression of G proteins, subunits that are susceptible to pertussis toxin. To this aim, we will transduce endothelial cells with several Ga shRNA Lentiviral particles, and measure inflammatory cytokine secretion as a functional read-out under high glucose in the presence or absence of C-peptide. We expect that the anti-inflammatory effect of C-peptide (with decrease in cytokine secretion) not be present in cells transduced with shRNA Lentiviral particle that will knock down one of the Ga subunits.

Our second quarterly scientific progress report for the initial year of our project (12/28/10 – 03/27/11) described the following:

C-peptide exerts beneficial effects on the vascular endothelium exposed to high glucose by decreasing apoptosis (Specific Aim 3).

T1D is a well-established risk factor for vascular disease. Chronic elevations of blood glucose level (hyperglycemia) and systemic low- grade inflammation contribute to the development of endothelial dysfunction, an early event in the pathogenesis of vascular disease in diabetes. High glucose damages endothelial cells by increasing oxidative stress through generation of reactive oxygen species (ROS) and inducing apoptosis. ROS are powerful cellular activators of the nuclear-factor(NF)-kB pathway, which regulates activation of a series of cytokine and adhesion molecule genes that results in the adhesion of leukocytes to endothelial cells and release of cytotoxic molecules. In human aortic endothelial cells, activation of NF-κB accelerates apoptosis by downregulating expression of Bcl-2, an anti-apoptotic factor.

C-peptide, the cleavage product of the proinsulin molecule in the pancreatic beta-cells of the pancreas, has been shown to exert insulin-independent biological effects on a number of cells proving itself as a bioactive peptide with anti-inflammatory properties. Since T1D patients typically lack physiological levels of insulin and C-peptide, this is considered an important factor in the pathophysiology of diabetic complications. Recently, we have demonstrated that C-peptide improves endothelial dysfunction and decreased inflammation in a model of high glucose-induced endothelial dysfunction. In this study, we tested whether the beneficial effect of C-peptide on the endothelium also includes an inhibitory effect on high glucose-induced apoptosis.

Experiment 1. Detection of Apoptosis in high glucose-exposed endothelial cells in the presence of C-peptide. Human Aortic Endothelial Cells (HAEC) were seeded in 96-well plates and the next day treated with normal glucose medium (5.5mmol/L) or high glucose medium (25mmol/L) in the presence or absence of C-peptide (10nmol/L) or scrambled C-peptide as control for 48 h at 37°C. Apoptosis was detected using the Cell Death Detection ELISA^{PLUS} kit (Roche Diagnostics GmBH, Mannheim, Germany), which detects cytoplasmic histone-associated-DNA-fragments after induced-cell death. Three independent experiments were run in which each condition was tested in triplicate. Results were expressed as absorbance raw data (mean ± SD) and percentage of apoptosis versus high glucose condition.

As shown in **Figure 1A**, exposure of HAEC to high glucose medium (HG) for 48 h significantly increased apoptosis as compared to cells in normal glucose (NG; $p<0.01$). Addition of C-peptide (CP) to the high glucose medium significantly reduced HAEC apoptosis, as compared to high glucose alone ($p<0.01$), while scrambled C-peptide (Scr. CP) did not cause any significant decrease in apoptosis. This effect corresponded to a 25% reduction of apoptosis by C-peptide as compared to high glucose (**Figure 1B**).

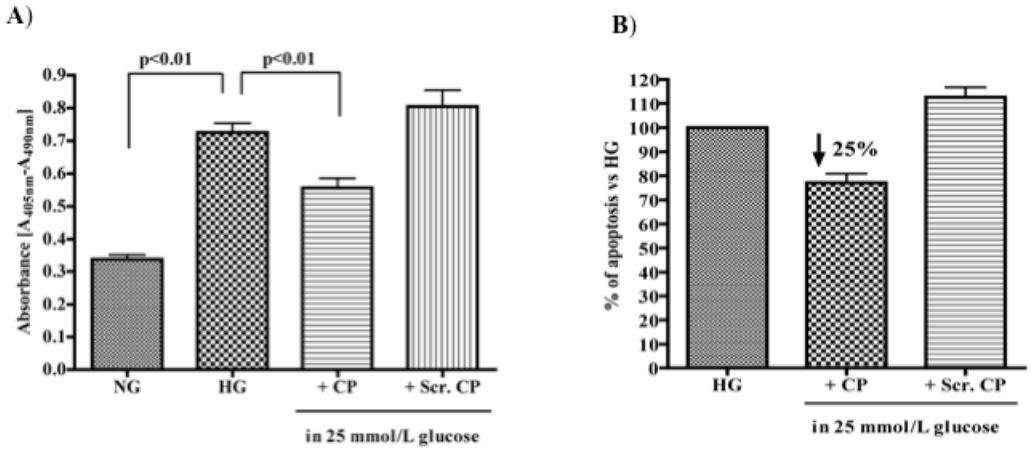

Figure 1

Experiment 2. Detection of caspase-3 protease expression and activity in high glucose-exposed endothelial cells in the presence of C-peptide. One crucial mediator of apoptosis is the activated caspase-3 protease, which catalyzes the specific cleavage of many key cellular proteins. We evaluated endogenous levels of the large fragment (17/19kD) of activated (cleaved) caspase-3 by western blotting in cytoplasmic cell lysates from HAEC exposed to high glucose overnight. Cytosolic and membrane proteins were extracted using Qproteame Cell Compartment kit (QIAGEN, Valencia, CA) and protein content was measured using a bicinchoninic acid assay kit (Pierce Biotechnology). Aliquots of protein extracts (30µg) were subject to immunoblot analysis using rabbit polyclonal anti-cleaved caspase-3 (1:500) (Cell Signaling Technology, Danvers, USA) and mouse monoclonal anti-β-actin antibody (1:10,000; Sigma). Densitometry was performed with UN-SCAN-IT gel software (Silk Scientific, Orem, UT). A minimum of three independent experiments was performed. As shown in **Figure 2A**, expression of activated caspase-3 was higher in cell lysates from HAEC under high glucose (HG) compared to normal glucose conditions (NG;$p<0.05$). Addition of C-peptide (CP) reduced endogenous levels of activated caspase-3 compared to high glucose alone ($p<0.05$), a result that was not observed with scrambled C-peptide (**Figure 2A**).

We assessed caspase-3 activity in cytoplasmic cell lysates from high glucose-exposed HAEC by ELISA. As shown in **Figure 2B**, exposure to high glucose medium overnight significantly increased caspase-3 activity in HAEC of 1.5 fold compared to normal glucose ($p<0.01$). Addition of C-peptide to the high glucose medium, significantly reduced caspase-3 activity ($p<0.01$ vs. high glucose alone), while scrambled C-peptide showed no significant effects (**Figure 2B**).

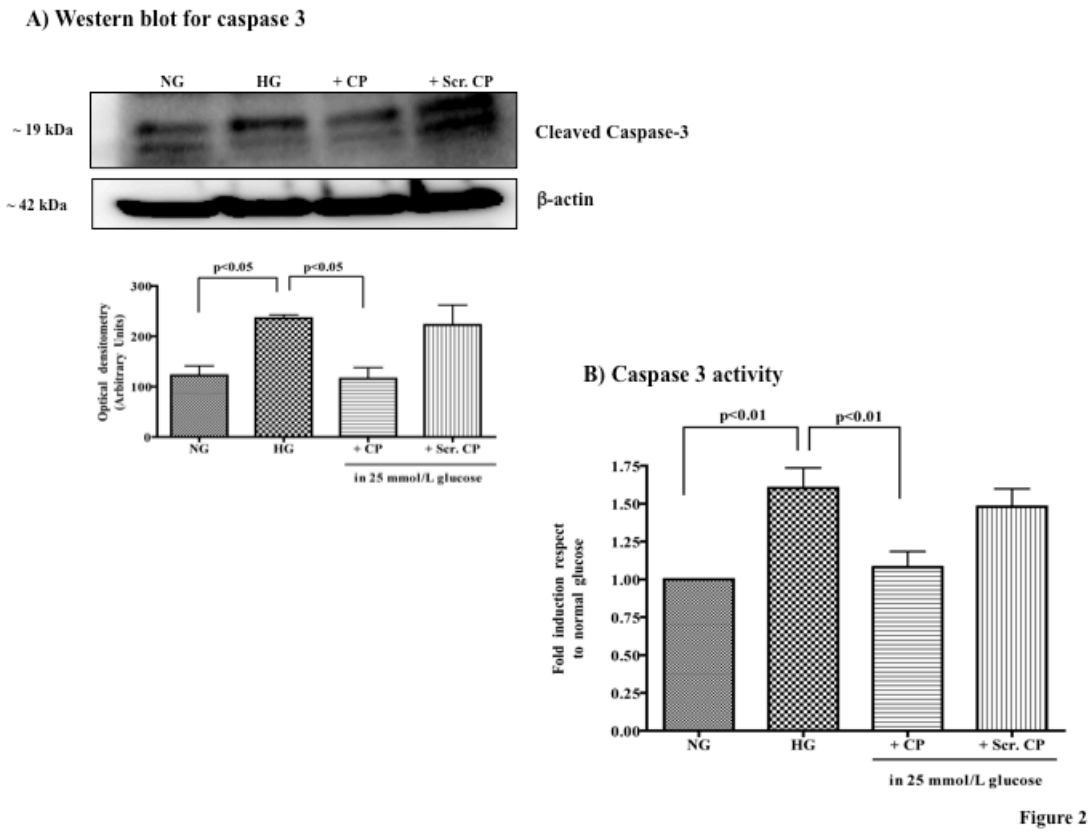

Figure 2

Experiment 3. Detection of the anti-apoptotic factor Bcl-2 in high glucose-treated HAEC in the presence of C-peptide. Analysis of expression of the product of the survival gene Bcl-2 by western blotting using a rabbit polyclonal anti-Bcl-2 antibody (Cell Signaling) showed that exposure of HAEC to high glucose (HG) overnight significantly decreased levels of Bcl-2 protein compared to normal glucose (NG) (**Figure 3**, $p<0.05$). Addition of C-peptide (CP) to the high glucose medium increased Bcl-2 protein expression to levels detected under normal glucose (**Figure 3**, $p<0.05$ vs. high glucose). A minimum of three independent experiments was performed.

Conclusions. With these series of experiments, we demonstrated that C-peptide reduced glucose-induced apoptosis of HAEC. Many cellular mediators of the apoptotic process, such as the caspase family, play an important role in the apoptotic process. Among this family, activation of caspase-3 is a central component of the proteolytic cascade in of glucose-exposed human endothelial cells. In our model, we found that overnight exposure to high glucose increased expression levels and activity of cleaved (activated) caspase-3, which was reduced by addition of C-peptide to HAEC *in vitro*. Moreover, expression levels of the anti-apoptotic molecule Bcl-2 were up-regulated by C-peptide as compared to high glucose alone in HAEC. Altogether, these findings support the view that C-peptide exerts an important biological activity in preventing cellular death by apoptosis.

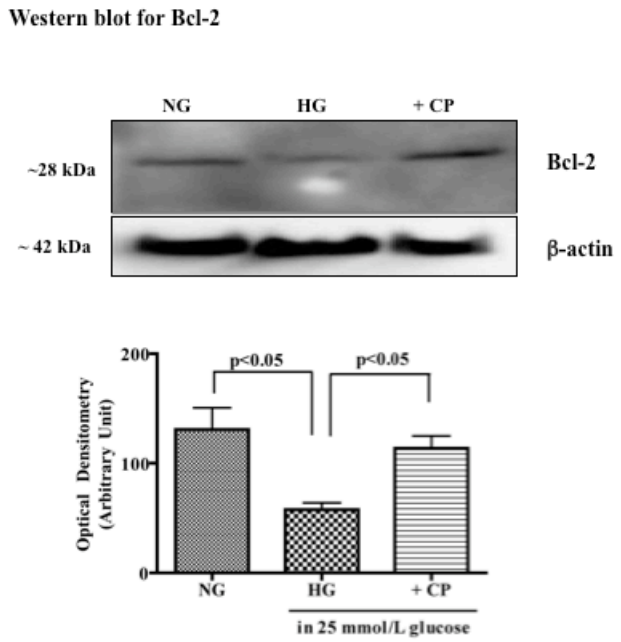

Figure 3

Experiment 4. C-peptide decreases ROS generation in HAEC by affecting NADPH oxidase enzymatic activity. We have previously shown that C-peptide decreases high glucose-induced ROS generation in HAEC. High glucose-induced ROS generation in endothelial cells mainly involves a NAD(P)H oxidase-dependent mechanism, which transfers electrons from NAD(P)H to molecular oxygen, producing O^{-}_{2}. Here, we tested whether C-peptide modulates activity of the NADPH oxidase enzyme in live cells using lucigenin-derived chemiluminescence. HAEC were seeded into T-25cm^2 flasks, serum starved overnight, and exposed to normal glucose medium or high glucose medium in the presence or absence of C-peptide for 30 minutes. NAD(P)H oxidase activity was also tested in HAEC pre-treated with the pharmacological inhibitors apocynin (10μmol/l; Sigma) and diphenyliodonium (DPI) (100μmol/l; Sigma) for 2 h. After detachment from the flasks, cells were resuspended in Krebs–Henseleit buffer (10 mmol/l glucose, 0.02 mmol/l Ca-Tritriplex, 25 mmol/l $NaHCO_3$, 1.2 mmol/l KH_2PO_4, 120 mmol/l NaCl, 1.6 mmol/l $CaCl_2{\cdot}2H_2O$, 1.2 mmol/l $MgSO_4{\cdot}7H_2O$, and 5 mmol/l KCl, pH 7.4) and seeded in white 96-well plates (10^5 cells/well). Superoxide anion production was measured in the presence of lucigenin, (5μmol/l, incubated for 20 min). The reaction was started by the addition of NAD(P)H (100μmol/l), and the relative light units (RLU) luminescence were measured over a period of 30 min in a Victor3 multi-well reader (PerkinElmer, Shelton, USA). Three experiments were performed in which each condition was tested in quadruplicate. Results were expressed as percent (mean ± SD) of NAD(P)H oxidase activity.

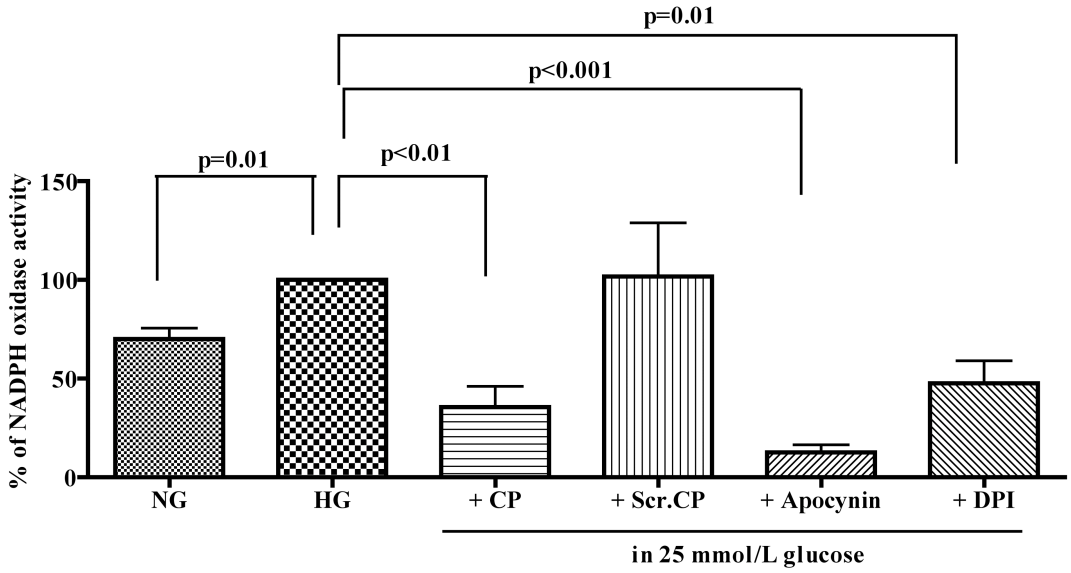

Figure 4

Exposure of HAEC to high glucose (HG) for 30 min increased NAD(P)H oxidase activity as compared to normal glucose (NG) (p=0.01). When C-peptide (CP) was added to the culture medium for 30 min, it significantly reduced NAD(P)H oxidase activity as compared to high glucose alone (p<0.01), while scrambled C-peptide (Scr. CP) did not have any significant effect (**Figure 4**). As expected, the specific pharmacologic NAD(P)H oxidase inhibitors DPI and apocynin significantly abolished high glucose-induced NAD(P)H oxidase activation in HAEC (p<0.01 and p=0.01, respectively).

Experiment 5. Effect of C-peptide on the Rac-1-pathway of NADPH oxidase activation. The NAD(P)H oxidase enzyme is composed of four functional components whose assembly requires the presence of the small GTP-binding protein Rac-1 at the plasma membrane. In endothelial cells, Rac-1 controls low-intensity basal superoxide production as well as bursts of NAD(P)H oxidase activity, such as during exposure to high glucose. In T1D diabetes, Rac-1-mediated ROS generation is considered an important pathophysiological pathway in the development of vascular complications. In a recent report, it was shown that glucose induced-NAD(P)H oxidase activation, inflammatory responses, and cardiovascular complications were attenuated in an animal model of Rac-1 knockout. This suggests that targeting inhibition of Rac-1 may represent an attractive therapeutic approach for reducing inflammatory-induced vascular damage in diabetes.

We investigated Rac-1 protein level of expression in the cytoplasm and plasma membrane of high glucose-exposed HAEC by western blotting. Cytosolic and membrane proteins were extracted using Qproteame Cell Compartment kit (QIAGEN, Valencia, CA) and protein content was measured using a bicinchoninic acid assay kit (Pierce Biotechnology). Aliquots of protein extracts (30µg) were subject to immunoblot analysis using rabbit polyclonal anti-Rac-1 (1:1000). Densitometry was performed with UN-SCAN-IT gel software (Silk Scientific, Orem, UT). A minimum of three independent experiments was performed. As showed in **Figure 5A**, exposure of HAEC cells to high glucose (HG) for 30 min triggered translocation of Rac-1 from the cytoplasm (Rac-1-c) to the plasma membrane (Rac-1-m) as compared to exposure to regular medium (NG; p<0.05). Addition of C-peptide (CP) to high glucose medium for 30min, significantly reduced Rac-1 translocation from the cytoplasm to the membrane (p<0.05 vs. high glucose) (**Figure 65A**).

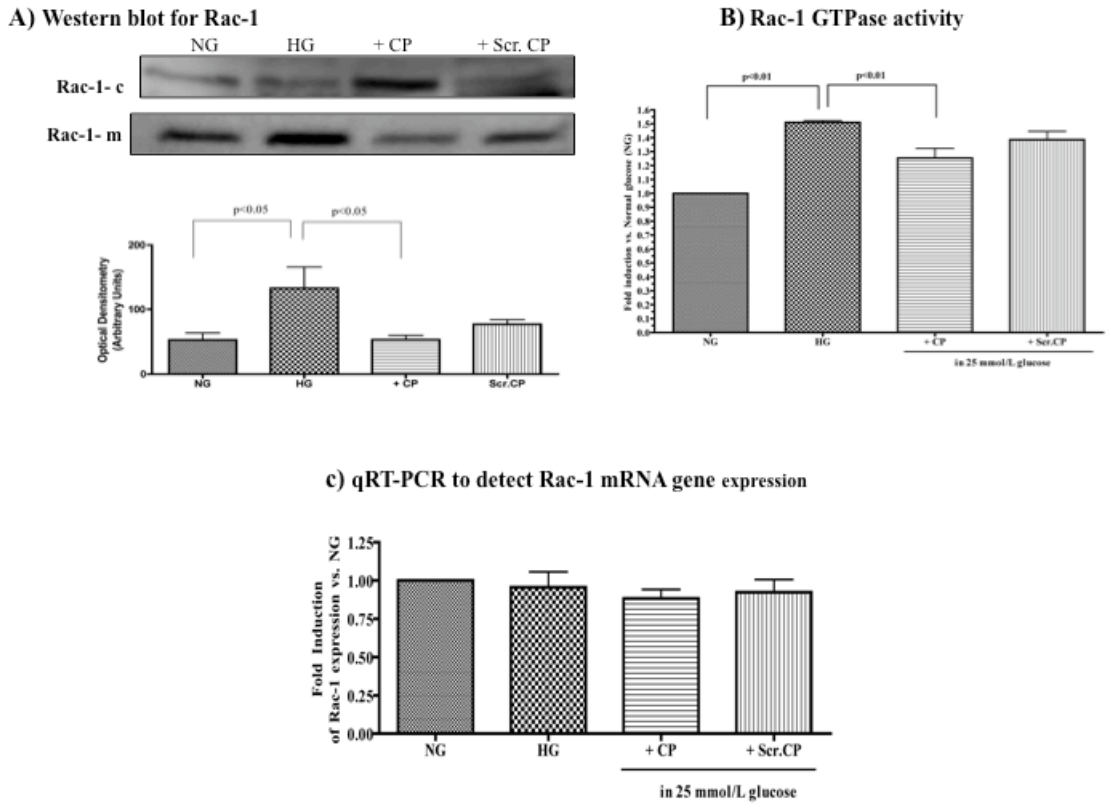

Figure 5A

Rac is a member of the Rho family of small GTP-ases that undergoes regulatory control by alternating between binding GTP for activation and hydrolysis to GDP for inactivation. We investigated whether intrinsic Rac-1 GTP-ase activity was affected by C-peptide. Briefly, HAEC were grown into T-75cm^2 flasks, serum starved overnight and exposed to treatment conditions for 30 min. Rac GTPase activity was measured in 10μg of cell lysates using a commercially available Rac G-LISA^{TM} Activation Assay kit which measures the GTP form of Rac from HAEC lysates following manufacturer's instructions (Cytoskeleton, Inc, Denver, CO). At least 4 experiments were run in which each condition was tested in duplicate. Results are expressed as GTPase activity-fold induction (mean ± SD) compared to normal glucose condition. In **Figure 5B**, HAEC exposed to high glucose medium (HG) for 30 min significantly increased GTP-ase activity compared to regular glucose medium (NG;$p<0.01$). When C-peptide (CP) was added to the high glucose medium, we observed a significantly reduced GTP-ase activity in HAEC cells as compared to high glucose alone ($p<0.01$). On the contrary, scrambled C-peptide (Scr. CP) did not significantly affect GTPase activation.

We tested whether C-peptide treatment for 30 min had any effects on mRNA gene expression of Rac-1 in high glucose-exposed HAEC. Briefly, HAEC were grown into T-75cm^2 flasks, then were serum starved overnight and exposed to treatment conditions for 30 min. Total RNA was isolated using RNAqueous-4PCR kit (Ambion, Austin, TX) and quantified by spectrophotometry. One microgram of RNA was reverse transcribed to cDNA (5 min at 65°C, 50 min at 50°C and 5 min at 85°C) using oligo(dT) primers (Invitrogen, Carlsband, CA). Using the LightCycler system (Roche Diagnostics), quantitative real time PCR was performed using the following primers: *Rac-1* sense 5' AGGAAGAGAAAATGCCTG-3' and antisense 5'-AGCAAAGCGTACAAAGGT-3' and housekeeping gene *GAPDH* sense 5'-TCGGAGTCAACGGATTTGGTCGTA-3' and antisense 5'-TGGCATGGACTGTGGTCATGAGTC-3'. Aliquots of the cDNA were loaded into capillary tubes and amplified for 40 cycles. The PCR products were further analyzed by agarose gel electrophoresis to

confirm the correct length of the amplified products. Rac-1 data were normalized using GAPDH housekeeping gene and results were expressed as fold induction versus normal glucose condition (mean ± SD of three independent experiments). As shown in **Figure 5C**, we did not find any significant differences in mRNA expression for Rac-1 in HAEC exposed to the different conditions tested.

Conclusions. We showed that C-peptide inhibits glucose-induced NAD(P)H oxidase activation, which is the major source of ROS in endothelial cells. This multi-component enzyme includes a membrane-bound cytochrome b_{558}, comprised of $p22^{phox}$ and $gp91^{phox}$ subunits, and the cytosolic adapter proteins $p47^{phox}$ and $p67^{phox}$, which are recruited to the cytochrome during stimulation to form a catalytically active oxidase. Recruitment of $p47^{phox}$ and $p67^{phox}$ to the plasma membrane, requires presence of Rac-1, a member of the Rho family of small GTP-binding proteins, which complex with the cytosolic proteins to regulate NAD(P)H oxidase activity. In this study, we report that glucose-induced Rac-1 protein levels at the plasma membrane of HAEC were reduced by 30 min treatment with C-peptide *in vitro*. Moreover, glucose-induced Rac GTPase activity was also reduced by C-peptide in HAEC. All together, these findings demonstrate that C-peptide decreases ROS generation by affecting Rac-1-dependent NAD(P)H oxidase activation in glucose-exposed HAEC. How exactly C-peptide interferes with Rac-1-mediated NAD(P)H generation of ROS is not known. Based on our data, we support the hypothesis that C-peptide may interfere with translocation of Rac-1 from the cytoplasm to the membrane. In fact, membrane expression of Rac-1 and its GTPase activity were significantly reduced in C-peptide-treated endothelial cells. In our model, no effect by C-peptide on Rac-1 mRNA gene expression was detected after 30 min exposure. Thus, we conclude that C-peptide may have an effect on post-translational modifications (i.e., isoprenylation) of Rac-1 that are required for translocation to the plasma membrane upon activation. In addition, C-peptide may also affect translocation of the other NAD(P)H cytoplasmic subunits $p67^{phox}$ and/or $p47^{phox}$ which, when bound to Rac-1, can migrate from the cytoplasm to plasma membrane where activation of the cytochrome occurs. Further studies are necessary to investigate these alternatives.

Thus, based on these findings we suggest that in healthy individuals C-peptide may represent an endogenous molecule with antioxidant properties that, once secreted in the bloodstream, protects the vascular endothelium from the damaging effects of hyperglycemia-induced oxidative stress.

12. Use additional page(s) to present a brief statement of plans or milestones for the next quarter.

Our efforts are concentrated on the experiments aimed at isolating the C-Peptide Receptor (CPR) as presented in our original Aims 1 and 2. These experiments are ongoing in the laboratory right now.

1. **Pull-down experiments:** We have used a set of biotinylated C-peptides, including a wild-type and a randomized version of C-peptide, in pulled-down experiments in an effort to isolate the specific proteins that will be interacting with C-peptide following its physiological internalization in early endosomes. We expect that the proteins that are pulled-down by the biotinylated wild-type C-peptide be different that the ones from the scrambled C-peptide probe. Since the last Progress Report we have perfected the procedure for endosome isolation from HEK and endothelial cells and performed several experiments of pull-down. The interacting proteins have been sent to Sequencing Facilities for protein ID. We are constantly improving the experimental procedure of these experiments by cross-linking the C-peptide to the plasma membrane so that the binding with the hypothetical CPR will be stronger and more resistant to the different procedures of isolation.

2. **siRNA experiments:** It has been suggested that the CPR is a G-protein-coupled receptor from studies in which pertussis toxin inhibits the binding to cell membrane and some intracellular signaling. Based on these published results, we have knocked down gene expression of G proteins a subunits that are susceptible to pertussis toxin. To this aim, we have transduced endothelial cells with several Ga shRNA Lentiviral particles, and measured inflammatory cytokine secretion as a functional read-out under high glucose in the presence or absence of C-peptide. We have generated preliminary results in which we identify some Ga subunits as those involved in the anti-inflammatory effect of C-peptide. As an

important control, we are now checking with western blot whether we were successful in knocking down gene expression of all the G proteins a subunits that we are studying. If so, we can certainly conclude that a specific Ga protein is mechanistically responsible for the inhibitory effect of C-peptide on cytokine secretion under high glucose.

3. Microarray experiments: As an additional approach to help identification of the CPR, we have also performed microarray experiments in C-peptide treated and not treated endothelial cells to see whether certain G-protein associated genes are up-regulated or down-regulated by C-peptide. The Bioinformatics Core of the University of Pittsburgh is now analyzing the results from 5 independent experiments performed.

In the third quarterly scientific progress report (03/28/11 - 06/27/11) of year 01, we now report on our cumulative results.

The paper, in which we summarized all the results presented in our Second Quarterly Report, was submitted to *Diabetologia* for publication. Although the reviewers have found our paper interesting and deserving publication, they suggested some additional experiments to make our points stronger and our conclusions more convincing.

Their suggestions (and our answers) will be hereafter presented in the order they were formulated.

1. ***Does the effect of C-peptide also extend the induction of apoptosis by other agents or does it only reduce glucose-induced apoptosis?***

To answer to this comment, we have investigated the effect of C-peptide on tumor necrosis factor (TNF)-α-mediated apoptosis of endothelial cells. TNF-α is an inflammatory cytokine that induces apoptosis and has been implicated in the pathogenesis of diabetic micro-vascular complications. In agreement with Al-Rasheed NM *et al.* [*Al-Rasheed NM, Willars GB, Brunskill NJ (2006) C-peptide signals via Galpha I to protect against TNF-alpha-mediated apoptosis of opossum kidney proximal tubular cells. J Am Soc Nephrol 17:986-995*] we observed that TNF-α-induces apoptosis of endothelial cells by increasing DNA fragmentation and cleaved Caspase-3 expression as compared to medium without TNF-α. C-peptide significantly reduced DNA fragmentation, decreased level of cleaved Caspase-3 and increased Bcl-2 protein expression levels in endothelial cells. We have then added text in the manuscript in the Materials and Methods, Results, and Discussion sections. A new Figure 5 shows these results.

Materials and Methods

Detection of TNF-α-mediated apoptosis

HAEC were seeded in 96-well plates and the next day treated with either regular EBM-2 alone, or EBM-2 with TNF-α (20ng/mL*),* or EBM-2 with TNF-α (20ng/ml) in the presence of 10nmol/l C-peptide for 24 h. In this latter condition, HAEC were pre-treated with 10nmol/l C-peptide for 24 h before adding TNF-α*.* Apoptosis was detected using the Cell Death Detection ELISA^{PLUS} kit (Roche Diagnostics GmBH), as above. For Bcl-2 and cleaved Caspase-3 proteins detection we followed the same methodology as previously described for immuno-blotting. Three independent experiments were run. ELISA results were expressed as absorbance raw data (mean ± SD).

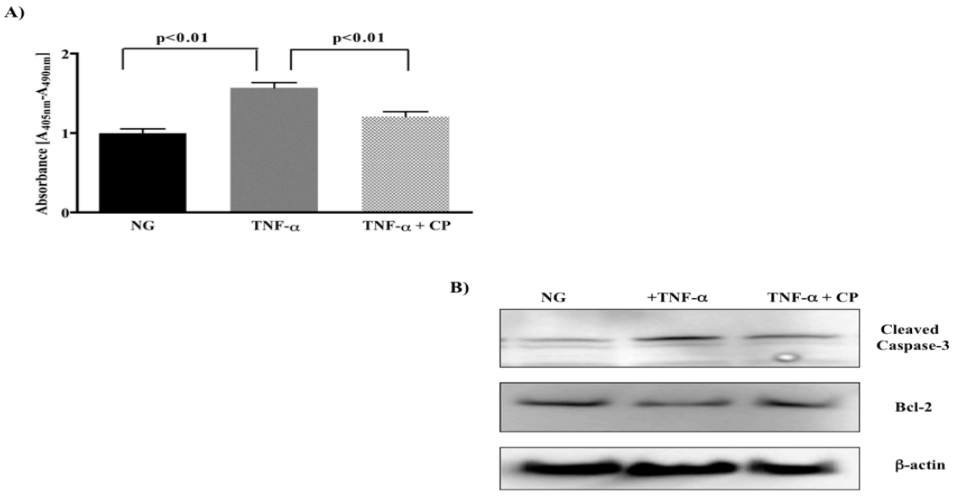

Figure 5

Figure 5. C-peptide antagonizes TNF-a-mediated apoptosis of HAEC. HAEC were exposed to normal glucose (NG) medium with or without TNF-a (20ng/ml) in the presence or absence of C-peptide (CP; 10nmol/l) for 24 h. In **A**) Changes in cytoplasmic histone-associated-DNA fragments in the different treatment conditions were detected using Cell Death Detection ELISA^{PLUS}. Significant increase of apoptosis was observed in TNF-a-exposed HAEC compared to NG alone (p<0.01). Addition of CP significantly reduced TNF-a-induced apoptosis as compared to HAEC cells exposed to TNF-a- alone (p<0.01). Results are expressed as mean ± SD (n= 3). In **B**) Representative image of immunoblots showing cleaved caspase-3 expression levels in HAEC exposed to the different conditions as above. While endogenous cleaved caspase-3 levels increased after exposure to TNF-a compared to exposure to medium alone, addition of C-peptide decreased caspase-3 levels. Bcl-2 protein levels in HAEC decreased after exposure to TNF-a-. Addition of C-peptide increased Bcl-2 expression to levels detected in normal glucose.

Results

C-peptide reduces TNF-α-mediated apoptosis of HAEC

As an additional model of endothelial cellular apoptosis, we investigated the one mediated by the inflammatory cytokine TNF-α, which play an important role in the development of diabetic complications. Exposure of HAEC to TNF-α for 24 h significantly increased endothelial cell apoptosis as compared to cells in normal glucose (p<0.01), as shown in Figure 4A. Addition of 10nmol/L C-peptide to TNF-α significantly reduced apoptosis, as compared to TNF-α alone (p<0.01) (Figure 5A).

As shown in Figure 5B, levels of activated caspase-3 was higher in cell lysates from HAEC treated with TNF-α compared to normal glucose conditions. Addition of C-peptide reduced endogenous levels of activated caspase-3 to expression levels observed under normal glucose (Figure 5B). Analysis of expression of the product of the survival gene Bcl-2 by western blotting showed that TNF-α treatment decreased Bcl-2 protein levels compared to normal glucose (Figure 5B). Addition of C-peptide reversed this condition by increasing Bcl-2 expression to levels detected under normal glucose (Figure 5B).

Discussion

Finally, in this paper we showed that C-peptide was able to decrease HAEC apoptosis induced by exposure to the inflammatory cytokine TNF-a, a result that was accompanied by a reduced expression of caspase-3 and up-regulation of Bcl-2. A similar result was reported few years ago by Al-Rasheed *et al.,* who showed that in kidney proximal tubular cells C-peptide protects from TNF-a-induced apoptosis. Altogether, these findings support the view that C-peptide exerts an important biological activity in preventing cellular death by apoptosis mediated by different inflammatory stimuli.

Although it has been shown that C-peptide acts *via* Ga_I possibly *via* a G-protein-coupled receptor to protect against TNF-a-induced apoptosis in kidney proximal tubular cells, the intracellular mechanisms of C-peptide-mediated anti-apoptotic effects in endothelial cells are not well understood. In high glucose-exposed endothelial cells, cellular apoptosis involves oxidative stress-triggered activation of the NF-kB pathway, which in turn suppresses Bcl-2 levels and activates Caspase-3 activity. We have previously observed that C-peptide interferes with glucose-induced nuclear translocation of the NF-kB p65/p50 subunits in HAEC, and reduces endothelial dysfunction. An effect of C-peptide on NF-kB and consequent decreased inflammatory cytokine production has also been reported in the brain of diabetic BB/Wor rats and found to be associated with reduced neuronal apoptosis. Here, we add significant pieces of information to this picture, by showing that C-peptide decreases intracellular ROS generation, a crucial upstream signaling event in the NF-kB pathway. In our model, ROS generation in HAEC was measured after overnight incubation with high glucose. C-peptide treatment quenched high glucose-induced ROS production closer to levels detected in normal glucose at all time points, reaching statistical significance at 3 h, thus suggesting that C-peptide exerts its beneficial effects on glucose-exposed endothelial cells over time.

2. Does C-peptide have an effect on the expression of the pro-apoptotic protein Bax?

Together with investigating C-peptide effect on Bcl-2 protein levels, we have also analyzed effect on the pro-apoptotic protein Bax by western blot. Since we did not found any significant effect of C-peptide on protein levels of Bax as compared to HAEC treated with high glucose alone, we decided of not showing the result in the manuscript. However, since the Reviewer asked for Bax, we included the image of the

Bax' immunoblot in Figure 4 as 4B and added the result in the text accordingly. Our results support those from Bugliani *et al.* [*Bugliani M, Torri S, Lupi R, Del Guerra S, Grupillo M, Del Chiaro M, et al. (2007) Effects of C-peptide on isolated human pancreatic islet cells. Diab/Metab Res Rev 23:215-219*] who reported a protective effect of C-peptide on apoptosis of human pancreatic beta-cells that was independent of Bax but promoting expression of the survival protein Bcl-2.

Materials and Methods

Immunoblotting for Bax, Bcl-2, cleaved caspase-3, and Rac-1

For Bax, Bcl-2 and cleaved caspase-3 proteins detection, HAEC were grown into T-75cm^2 flasks and exposed overnight to the treatment conditions as above. For Rac-1 protein detection, HAEC were serum starved overnight before exposing to the treatment conditions for 30 min. Cytosolic and membrane proteins were extracted using Qproteame Cell Compartment kit (QIAGEN, Valencia, CA) and protein content was measured using a bicinchoninic acid assay kit (Pierce Biotechnology). Aliquots of protein extracts (30µg) were subject to immunoblot analysis using rabbit polyclonal anti-Rac-1 (1:1000), anti-cleaved caspase-3 (1:500), anti-Bcl-2 antibodies (all from Cell Signaling Technology, Danvers, USA) and mouse monoclonal anti-β-actin antibody (1:10,000; Sigma). A rabbit polyclonal antibody anti-Bax (1:500) (Millipore, Billerica, MAas) was used to detect Bax protein levels. Densitometry was performed with UN-SCAN-IT gel software (Silk Scientific, Orem, UT). A minimum of three independent experiments was performed.

Results

C-peptide increases expression of the anti-apoptotic factor Bcl-2 in high glucose-treated HAEC

Analysis of expression of the product of the survival gene Bcl-2 by western blotting showed that exposure of HAEC to high glucose overnight significantly decreased levels of Bcl-2 protein expression to 50% of the levels detected in normal glucose (Figure 4A, $p<0.05$). Addition of C-peptide to the high glucose medium increased Bcl-2 protein expression to levels detected under normal glucose (Figure 4A, $p<0.05$ vs. high glucose). C-peptide did not change expression levels of the pro-apoptotic molecule Bax in glucose-exposed HAEC as compared to cells exposed to high glucose alone (Figure 4B).

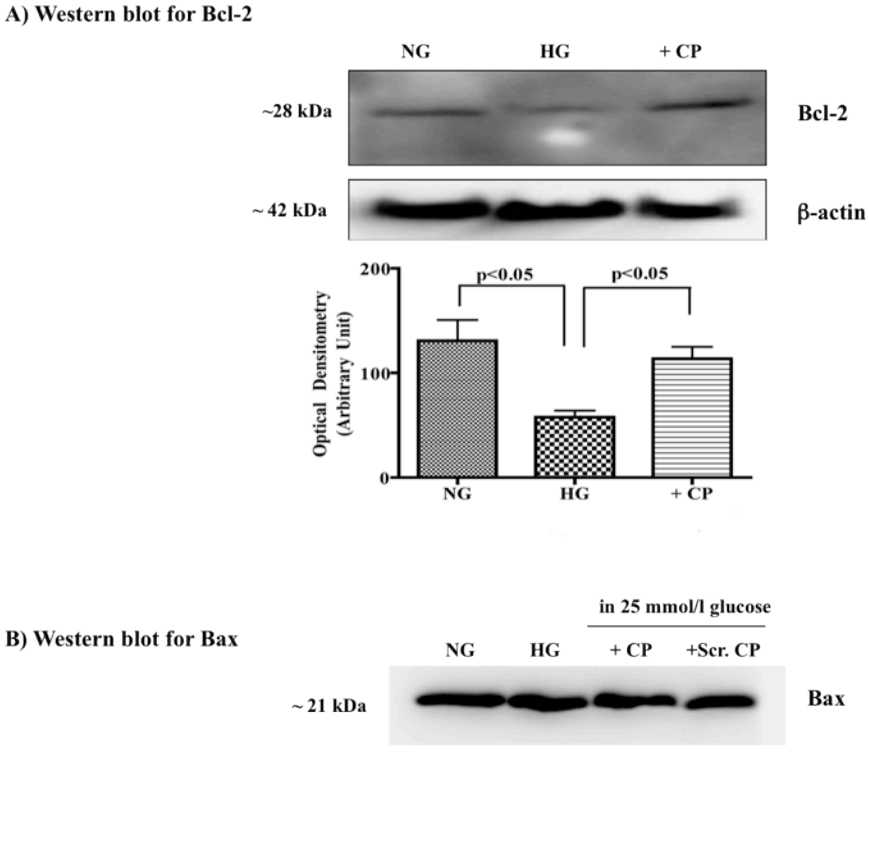

Figure 4

Figure 4. C-peptide increases Bcl-2 expression levels in HAEC exposed to high glucose. In **A**) Immunoblot of Bcl-2 and β–actin in extracts from HAEC in normal glucose (NG); high glucose (HG; 25mmol/L); or HG+10nmol/l C-peptide (CP) overnight. Densitometric quantitation of the bands showed that Bcl-2 levels in HG-exposed cells were significantly lower than in NG (p< 0.05). Addition of CP triggered an increase in levels of Bcl-2 (p<0.05 vs HG). Results are expressed as mean ± SD (n= 3). In **B**) Representative immunoblot of the pro-apoptotic molecule Bax in extracts from HAEC in NG; HG (25mmol/L); or HG+ either 10nmol/l CP or Scr. C-peptide (Scr. CP) overnight. C-peptide did not change expression of Bax in glucose-exposed HAEC.

Moreover, in agreement with Bugliani *et al.*, who studied human pancreatic beta-cells, expression levels of the anti-apoptotic molecule Bcl-2, but not of the pro-apoptotic molecule Bax, was up-regulated by C-peptide as compared to high glucose alone in HAEC. Bax belongs to the Bcl-2 protein family of apoptosis-regulator gene products that may function as apoptotic activators (Bax, Bak, Bad, and others) or facilitating cell survival (Bcl-2, Bcl-XL, Bcl-w, and others). Although the protective effect of C-peptide in endothelial cell apoptosis is reported here for the first time, the anti-apoptotic effect of C-peptide has been already described in different cellular models.

3. ***The authors should employ a second method to detect cell apoptosis to bolster their results.***

We have performed a TUNEL assay on glucose-exposed HAEC and investigated effect of C-peptide. The results of this assay were presented in the new Figure 2.

Materials and Methods

Detection of Apoptosis

HAEC were seeded in 96-well plates and the next day treated as above for 48 h. Apoptosis was detected using the Cell Death Detection ELISA^{PLUS} kit (Roche Diagnostics GmBH, Mannheim, Germany), which detects cytoplasmic histone-associated-DNA-fragments after induced-cell death. Three independent experiments were run in which each condition was tested in triplicate. Results were expressed as absorbance raw data (mean ± SD).

The TUNEL assay was also used to detect apoptosis with the *In Situ* Cell Death Detection Kit, Fluorescein (Roche Diagnostics) that allows identification of individual apoptotic cells by labeling DNA breaks that occur at the early stages of apoptosis. Briefly, HAEC were seeded in MatTek plates (MatTek, Ashland, MA, USA), exposed for 96 h to the treatment conditions as above, and TUNEL assay performed according tp manufacturer's instructions. The label incorporated at the damaged sites of DNA was visualized by a confocal fluorescent microscopy (OLYMPUS FLUOVIEW PV1000, Center Valley, PA, USA) at 40x magnification. Three independent experiments were run.

Results

Glucose-induced endothelial cell apoptosis was also evaluated by TUNEL assay under a confocal fluorescent microscopy (Figure 2). As compared to normal glucose, HAEC exposed to high glucose demonstrated a significant induction of cell apoptosis. However, C-peptide treatment (10nmol/l) decreased glucose-induced apoptosis. Scrambled C-peptide appeared to be without any significant effects as compared to high glucose alone (Figure 2).

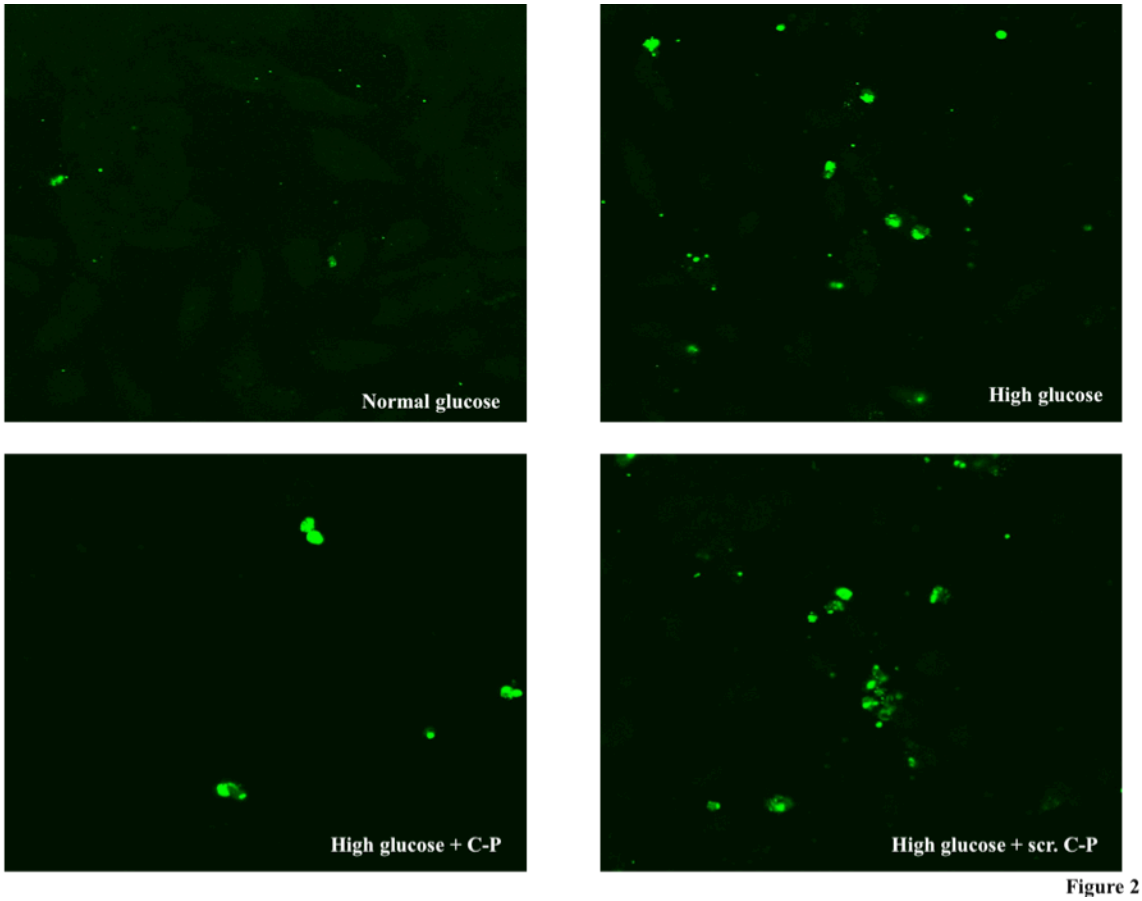

Figure 2

Figure 2. TUNEL assay in HAEC cultures. TUNEL stain showed an increase of apoptotic cells in HAEC exposed for 96 h to high glucose compared to normal glucose. C-peptide reduced the number of $TUNEL^{+}$ cells compared to high glucose alone. Scr. C-peptide had no significant effect. (40x magnification). Shown are representative images of 3 independent experiments.

4. ***Is the effect of C-peptide on apoptosis concentration-dependent?***

In response to this question, we tried a range of C-peptide concentrations (2nmol/l-50nmol/l) on endothelial apoptosis and observed that there was an increase in anti-apoptotic effect from 2 to 10nmol/l C-peptide concentrations, after which no effect was detected. These results are now included in the new Figure 1A.

Results

C-peptide decreases high glucose-induced apoptosis of HAEC

Exposure of HAEC to high glucose medium for 48 hours significantly increased apoptosis as compared to cells in normal glucose ($p<0.01$), as shown in Figure 1A-1B. Addition of C-peptide in a range of concentration 2-50nmol/l showed a decrease in glucose-induced apoptosis of HAEC that reached statistical significance with the C-peptide concentration of 10nmol/l (Figure 1Aand 1B). This effect corresponded to a 25% reduction of apoptosis by C-peptide as compared to high glucose. Higher concentrations of C-peptide (20 and 50nmol/L) did not have any statistical significant effects on high glucose-induced apoptosis, as shown in Figure 1A. Therefore, we chose C-peptide at 10nmol/l concentration for all other experiments. In Figure 1B, addition of scrambled C-peptide (10nmol/l) to the high glucose medium did not cause any significant effects on HAEC apoptosis, thus suggesting that the beneficial effect observed was specific to C-peptide.

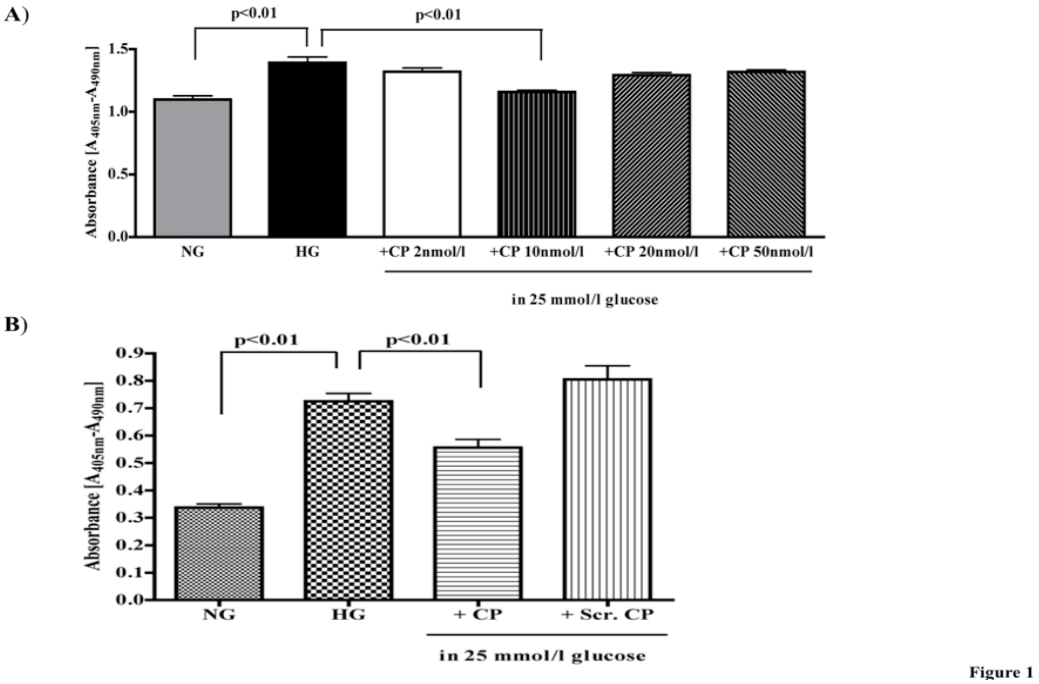

Figure 1

Figure 1. C-peptide decreases generation of histone-associated-DNA fragments in HAEC exposed to high glucose. In **A)** HAEC were exposed to normal glucose (NG) medium, or high glucose (HG;25mmol/l) medium in the presence or absence of a range of C-peptide (CP) concentrations and tested for changes in cytoplasmic histone-associated-DNA fragments in the different treatment conditions were detected using Cell Death Detection ELISA^{PLUS}. In B) HAEC were exposed to NG medium, or HG (25mmol/l) medium alone or with either CP (1onmol/l) or scrambled CP (scr. CP; 10nmol/l) for 48 h and tested for apoptosis as in **A**. A significant increase of apoptosis was found in HG-exposed HAEC compared to NG ($p<0.01$). C-peptide at 10nmol/l, but not Scr. CP, decreased apoptosis ($p< 0.01$ vs. HG). Higher concentrations of CP were not effective. Values are mean ± SD of three different experiments in which each condition was tested in triplicate.

With these additional experiments the paper was accepted for publication in ***Diabetologia,*** June 2011.

Cifarelli V, Geng X, Styche A, Lakomy R, Trucco M, Luppi P: C-peptide reduces high glucose-induced apoptosis of endothelial cells and decreases NAD(P)H-oxidase reactive oxygen species generation. **Diabetologia**, In press, 2011

12. Use additional page(s) to present a brief statement of plans or milestones for the next quarter.

Our efforts are now concentrated on the experiments aimed at isolating the C-Peptide Receptor (CPR) as presented in our original Aims 1 and 2. These experiments are ongoing in the laboratory right now.

1. **Pull-down experiments:** We have used a set of biotinylated C-peptides, including a wild-type and a randomized version of C-peptide, in pulled-down experiments in an effort to isolate the specific proteins that will be interacting with C-peptide following its physiological internalization in early endosomes. We expect that the proteins that are pulled-down by the biotinylated wild-type C-peptide be different that the ones from the scrambled C-peptide probe. Since the last Progress Report we have perfected the procedure for endosome isolation from HEK and endothelial cells and performed several experiments of pull-down. The interacting proteins have been sent to Sequencing Facilities for protein ID. We are constantly improving the experimental procedure of these experiments by cross-linking the C-peptide to the plasma membrane so that the binding with the hypothetical CPR will be stronger and more resistant to the different procedures of isolation.

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In the fourth quarterly scientific progress report (06/28/11 - 09/27/11) of year 01, we now report on our new and year 01 cumulative results.

C-peptide is the segment connecting insulin A and B chains. It is generated in pancreatic beta cells as the natural product of pro-insulin cleavage. For a long time, it was considered biologically important only for favoring pro-insulin folding within the secretory granules of the beta cells. Consistently with this view, the standard of care for diabetic, and especially T1D patients is solely insulin-replacement therapy; C-peptide is not administrated. However, recent studies have challenged this view. It has been offered increasing evidence that human C-peptide exerts intracellular effects in a variety of cells and could be of real benefit for diabetic patients who suffer from micro-vascular complications. How exactly C-peptide achieves these intracellular effects, however, is still unknown.

In previous experiments we have demonstrated that C-peptide reduces secretion of inflammatory cytokines from endothelial cells in a model of hyperglycemia-induced vascular injury by reducing activation of the nuclear factor (NF)-κB pathway (1). We found a similar anti-inflammatory activity of C-peptide in vascular smooth muscle cells (2). For the full-length, native, C-peptide, we found that, upon internalization from the cell surface, C-peptide quickly traffics to early endosomes and later proceeds to lysosomes for degradation (3). Trafficking of C-peptide to early endosomes is likely to account for its anti-inflammatory effects in vascular endothelial and smooth muscle cells. Based on these findings, it is hypothesized that C-peptide first binds to its cell surface receptor, then the complex internalizes and signals to effector pathways via endosomes (4). One major advance in this area would be the identification of the specific C-peptide receptor (CPR) at the level of the cellular membrane and characterization of C-peptide/CPR signaling to effectors upon internalization (5).

During this fourth quarter period our efforts to identify a C-peptide receptor have become more focused and refined. We have designed and obtained synthetic C-peptides that are biotinylated and incorporate a modified, photo-linkable leucine. This will allow us to capture molecules that bind C-peptide and have been cross-linked by UV irradation. We expect that this linkage will be critical for potential receptors that do not have the strong affinity that would be required to capture and carry them through the enrichment procedures, which are necessary for their characterization.

Sequence of Two Synthetized photo-linkable human C-peptides:

Biotin-**EAEDLQVGQVELGGGPGAGSLQPLALEGSLQ**
Biotin-**EAEDLQVGQVELGGGPGAGSLQPLALEGSLQ**

[Red **L** (Leucine) are L-Photo-Leucine]

First we determined the photo-Leucine amino acid half-life (Figure 1). Then, to validate our capture process we have been testing our modified C-peptides through their interaction with an anti-C-peptide antibody. We have been able to show that the C-peptide / anti-C-peptide antibody binding is sufficiently strong to capture the bound antibody via the biotin modified C-peptide even without cross-linking as revealed by SDS gel analysis. However, by western blot analysis only the UV treated samples retain biotin positivity after the SDS gel separation. We are now perfecting washing procedures to minimize the background proteins that are revealed on our gels and that may interfere with protein identification (Figure 2 and 3).

Photo-Leucine Crosslinking test.

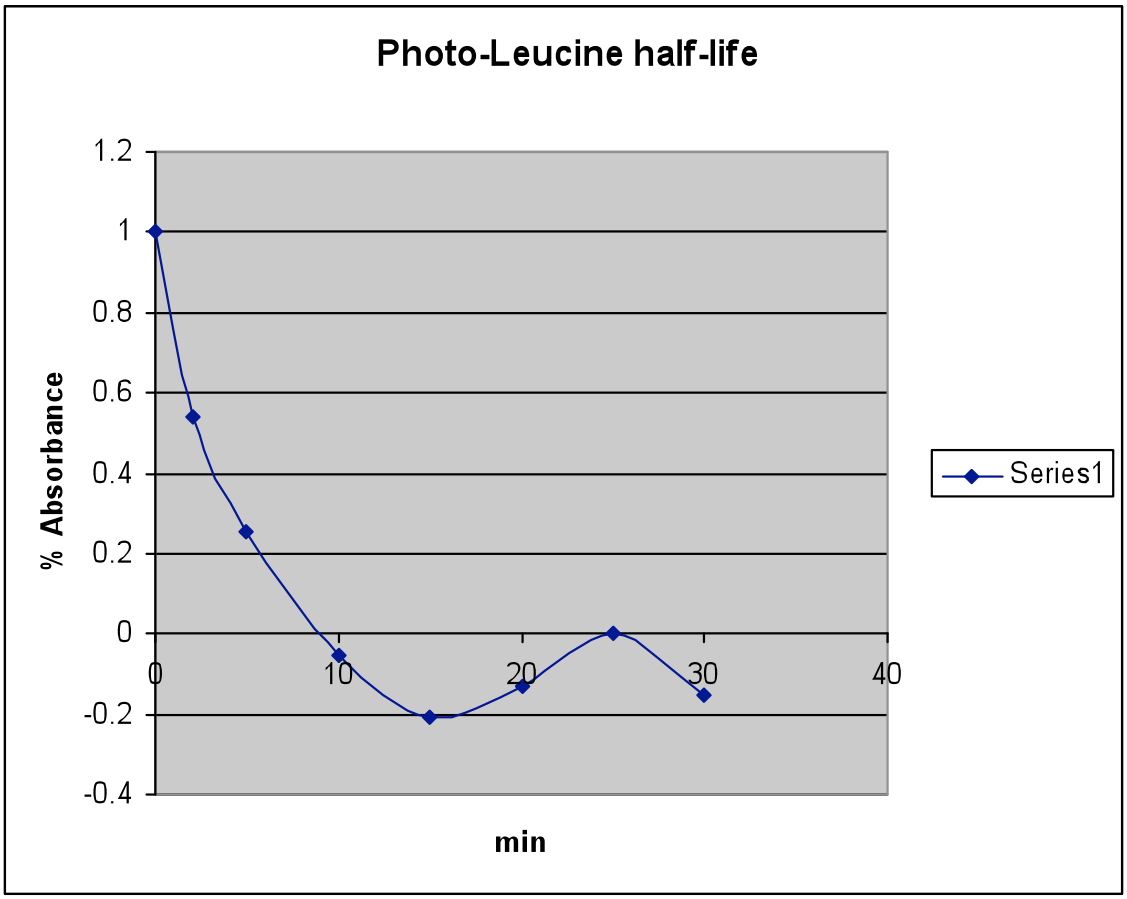

Figure 1. Photo-Leucine amino acid half-life determination: The sample of Photo-Leucine (1mg/ml) in PBS was irradiated UV (365nm) for 30mins, removing a portion of the sample every 5mins. The absorbance of each sample was measured spectro-photometrically at 345nm in comparison with blank with PBS, determining half of the Photo-Leucine activated.

Photo-C-peptide interacted with antibody, analyzed by SDS gel.

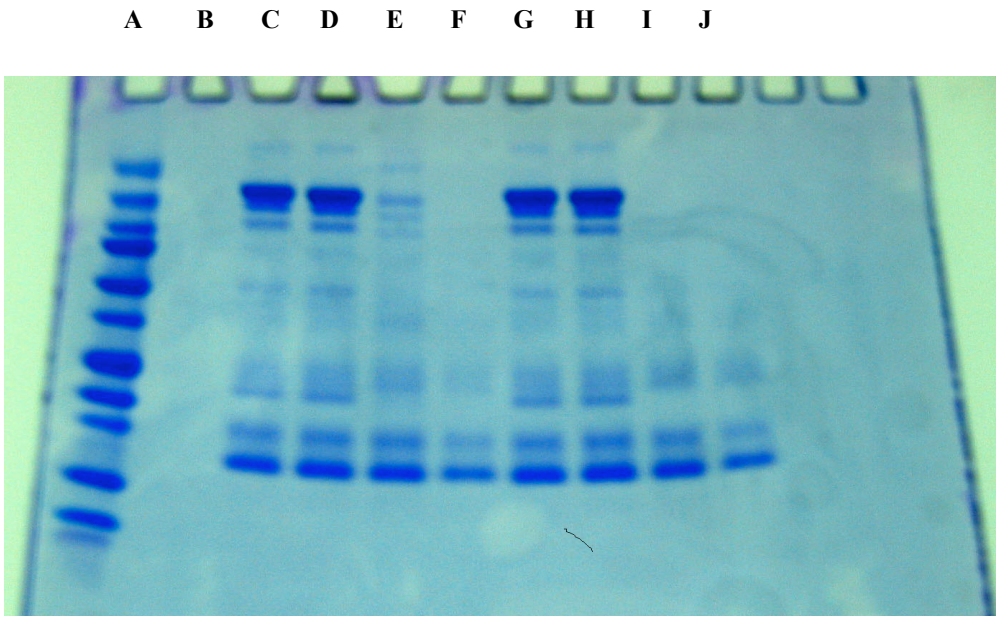

Figure 2. Photo-C-peptide interacted with antibody analyzed by SDS gel stain with commassie blue. **A**:Protein Marker;**B**:Empty;**C**:Photo-C-peptide add C-peptide antibody UV cross-linked;**D**: Photo-C-peptide add C-peptide antibody without UV irradiation;**E** Photo-C-peptide add control antibody (NCH4);**F**: Neutravidin bead control;**G,H,I** and **J** correspond with **C,D,E** and **E**. Its washed by 0.1% SDS in PBS.

Photo-C-peptide interacted with antibody, analyzed by Western blot.

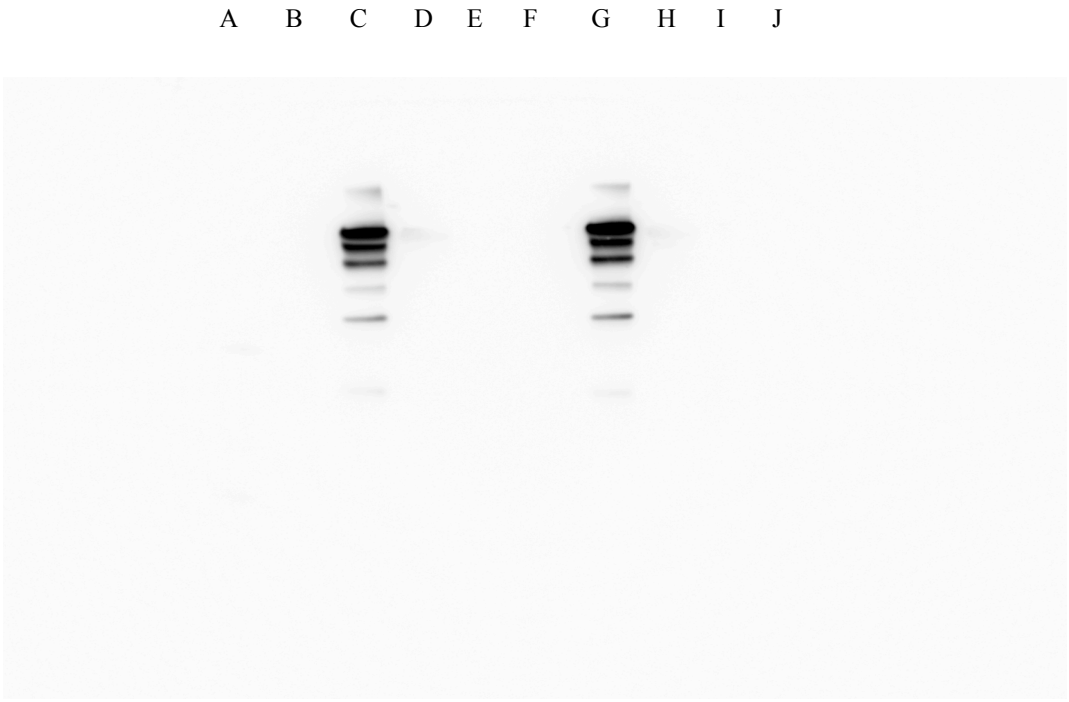

Figure 3. Photo-C-peptide interacted with antibody analyzed by Western Blot.
Same as **Figure 2**. **A:**Protein Marker;**B:**Empty;**C:**Photo-C-peptide add C-peptide antibody UV cross-linked;**D**: Photo-C-peptide add C-peptide antibody without UV irradiation;**E:** Photo-C-peptide add control antibody (NCH4);**F:** Neutravidin bead control;**G,H,I** and **J** correspond with **C,D,E** and **E**. washed by 0.1% SDS in PBS.

At the same time, we have been studying our modified C-peptides to determine that the modifications are not disrupting the biological responses normally observe with native C-peptide. We have been studying the effect of C-peptide on calcium flux in cultured, immortal cell lines that respond to C-peptide (Figure 4). This is the most direct and immediate effect seen in response to C-peptide. When we confirm responses to the modified C-peptides we also test for the calcium response in our endothelial cell cultures. Should any of these tests fail, we will design additional molecules with the cross-linkable amino acid at different positions of the C-peptide.

Once these steps are completed we will proceed with out attempts to capture the cross-linked, putative receptors from our endothelial cultures.

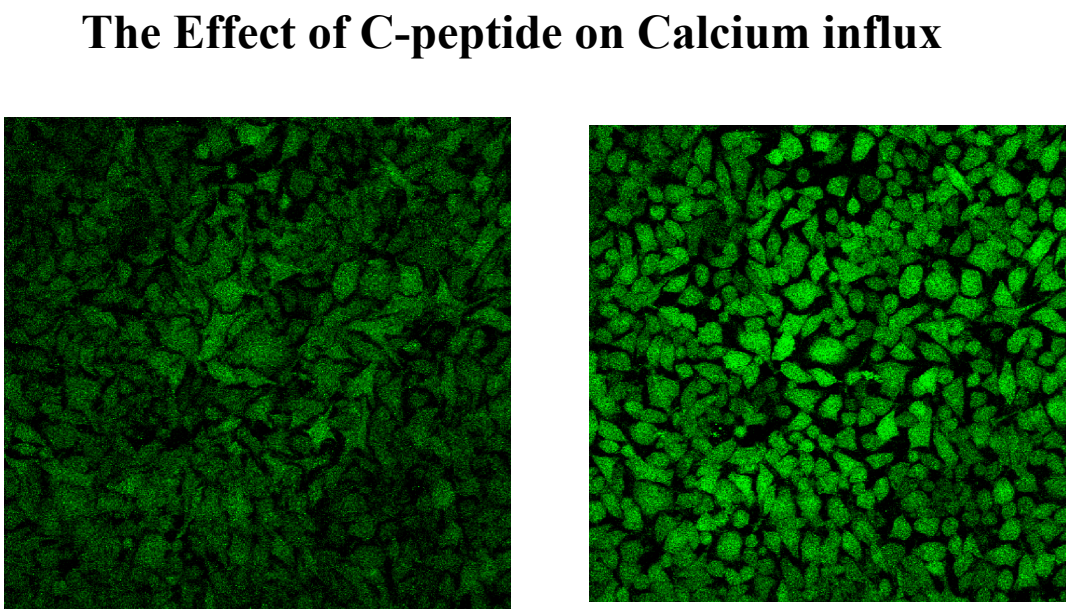

Figure 4.The effect of C-peptide on calcium influx. RAW264.7 cells were stained with 2uM Fluo-4-AM in culture media containing 2.5mM probenicid; 20 mM HEPES (PH7.3), 30min in room temperature.

A. Before add C-peptide; **B.** After add 100nM human C-peptide.

We have also begun studies of the *in vivo* effects of c-peptide. We are now testing our delivery system of C-peptide in mice. C-peptide is being continuously infused using an implanted osmotic pump (Figure 5) to deliver an amount of C-peptide equivalent to 0.25 U of insulin per day to diabetic mice that are also receiving insulin. We are monitoring circulating C-peptide to determine if our delivery process is effective and will make adjustments according to the results obtained over 30 days of treatment. The pumps are expected to function for about 30 days, so we anticipate replacing pumps at that interval to complete the 90 days of our actual experiments.

Figure 5

KEY RESEARCH ACCOMPLISHMENTS:

1) We have demonstrated that C-peptide reduces secretion of inflammatory cytokines fromendothelial cells in a model of hyperglycemia-induced vascular injury by reducing activation of the nuclear factor (NF)-κB pathway (Luppi P, Cifarelli V, Tse H, Piganelli J, Trucco M: Human C-peptide antagonizes high glucose-induced endothelial dysfunction through the NK-kB pathway. **Diabetologia** 51:1534, 2008).

2) We found a similar anti-inflammatory activity of C-peptide in vascular smooth muscle cells (Cifarelli V, Luppi P, Tse HM, He J, Piganelli J, Trucco M: Human proinsulin C-peptide reduces high glucose-induced proliferation and NF-kB activation in vascular smooth muscle cells. **Atherosclerosis** 201:248, 2008).

3) For the full-length, native, C-peptide, we found that, upon internalization from the cell surface, C-peptide quickly traffics to early endosomes and later proceeds to lysosomes for degradation (3. Luppi P, Geng X, Cifarelli V, Drain P, Trucco M: C-peptide is internalized in human endothelial and vascular smooth muscle cells via early endosomes. **Diabetologia** 52:2218, 2009).

4) Trafficking of C-peptide to early endosomes is likely to account for its anti-inflammatory effects in vascular endothelial and smooth muscle cells. Based on these findings, it is hypothesized that C-peptide first binds to its cell surface receptor, then the complex internalizes and signals to effector pathways via endosomes (Haidet J, Cifarelli V, Trucco M, Luppi P: Anti-inflammatory properties of C-peptide. **Rev Diabet Stud** 6:168, 2009).

5) One major advance in this area would be the identification of the specific C-peptide receptor (CPR) at the level of the cellular membrane and characterization of C-peptide/CPR signaling to effectors upon internalization (Cifarelli V, Trucco M, Luppi P: Anti-inflammatory effects of C-peptide prevent endothelial dysfunction in type 1 diabetes. **Immun Endoc & Metab Agents in Med Chem** 11:59, 2011).

REPORTABLE OUTCOMES:

Manuscripts (4 publications)

1. Cifarelli V, Trucco M, Luppi P: Anti-inflammatory effects of C-peptide prevent endothelial dysfunction in type 1 diabetes. **Immun Endoc & Metab Agents in Med Chem** 11:59, 2011.

2. Cifarelli V, Geng X, Styche A, Lakomy R, Trucco M, Luppi P: C-peptide reduces high glucose-induced apoptosis of endothelial cells and decreases NAD(P)H-oxidase reactive oxygen species generation. **Diabetologia** 54:2702, 2011.

3. Luppi P, Cifarelli V, Wahren J. C-peptide and long-term complications of diabetes. **Pediatric Diabetes** 2011: 12: 276–292.)

4. Haidet J, Cifarelli V, Trucco M, Luppi P: C-peptide reduces pro-inflammatory cytokine secretion in LPS-stimualted U937 monocytes in condition of hyperglycemia. **Inflammation Research**, In press, 2011

CONCLUSION:

Undoubtedly, there is much more to learn about C-peptide. Identification of the mechanism whereby C-peptide interacts with cell membranes, delineation of its intracellular signaling pathways in different cell types, and further evaluation of its transcriptional effects will enhance our understanding of C-peptide bioactivity. On the clinical side further studies of longer duration (>6 months) will be required to document the robustness of its beneficial effects on the different types of long-term complications in order to define its possible role in the therapy of T1D. Nevertheless, despite the fact that our knowledge is still incomplete, there are several lines of evidence in support of the notion that C-peptide is a bioactive peptide and that its replacement in T1D may be beneficial in the treatment of long-term complications. Even though the nature of the peptide's interaction with the cell membrane is only partially understood, its intracellular signaling characteristics and end effects including its action on eNOS, Na+,K+–ATPase, and several transcription factors are now well established for many cell systems and by different investigators. Results from studies in T1D patients and animal models demonstrate that C-peptide in replacement doses exerts beneficial effects on the early stage functional and structural abnormalities of both the kidneys and the peripheral nerves. The previous view that C-peptide is merely an inert by-product of insulin biosynthesis seems no longer tenable. Even a cautious evaluation of the available evidence presents the picture of a previously unrecognized bioactive peptide with therapeutic potential in an area where no causal therapy is available today. (See also Luppi P, Cifarelli V, Wahren J. C-peptide and long-term complications of diabetes. **Pediatric Diabetes** 2011: 12: 276–292.)

The So What Section:

What are the implication of this research?

Diabetes affects 16 million Americans and roughly 5-15% of all cases of diabetes are type 1 DM. It is the most common metabolic disease of childhood, and physicians diagnose approximately 10,000 new cases every year. Type 1 diabetes is associated with a high morbidity and premature mortality due to complications. The annual cost from diabetes overall exceeds $100 billion, almost $1 of every $7 dollars of US health expenditures in terms of medical care and loss of productivity. C-peptide administered regularly in physiological quantities might be an additive to insulin able to reduce those complications.

What are the military significance and public purpose of this research?

As the military is a reflection of the U.S. population, improved understanding of the underlying etiopathology of T1D will facilitate the development of potential therapeutics to prevent the onset of the disease or the development of diabetic complications among active duty members of the military, their families, and retired military personnel. Finding a cure to T1D will provide significant healthcare savings and improved patients' well being.

Anti-Inflammatory Effects of C-Peptide Prevent Endothelial Dysfunction in Type 1 Diabetes

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Abstract: C-peptide, historically considered a biologically inactive peptide, has been shown to exert insulin-independent biological effects on a number of cells proving itself as a bioactive peptide with anti-inflammatory properties. Type 1 diabetes (T1D) patients typically lack physiological levels of insulin and C-peptide. Recombinant insulin administrations, in the absence of C-peptide, correct hyperglycemia but leave an increased risk of developing microvascular complications affecting the small vessels of the eye, the kidneys, and the peripheral nerves. Inflammation is an important factor for the development of diabetes-associated vascular complications, and there is increasing evidence that T1D patients, even at a young age and after short duration of T1D, have circulating activated monocytes and increased plasma levels of inflammatory cytokines. It has been hypothesized that reduced plasma levels or lack of circulating C-peptide might contribute to the development of diabetes-associated vascular complications since C-peptide is able to reduce the inflammatory response associated with T1D. In this review, we present the most-up-to date information on the anti-inflammatory activity of C-peptide at the level of the vascular endothelium exposed to a variety of glucose levels. The anti-inflammatory properties of C-peptide in animal models of endotoxic shock and T1D-associated encephalopathy are also presented. The present evidence favours the view that T1D should be considered a dual-hormone deficiency disorder and emphasizes the possibility that replacement therapy with both insulin and C-peptide in T1D patients may offer an approach to retard or completely prevent the development of diabetes-associated microvascular complications, for which no causal therapy is available today.

Keywords: C-peptide, complications, diabetes, endothelium, inflammation, vascular.

INTRODUCTION

C-peptide is the 31 amino acid long peptide segment connecting insulin A and B chains, and a product of pro-insulin cleavage in the secretory granules, generated in the pancreatic beta cells as part of normal insulin production [1] (Fig. **1**). In healthy individuals, C-peptide is secreted in the portal and peripheral circulation in equimolar amount with insulin in response to elevated blood glucose, but it is absent in the majority of Type 1 diabetes (T1D) patients [2]. Once secreted, C-peptide circulates at low nanomolar concentrations, and it is primarily catabolized by the kidneys. Since C-peptide escapes hepatic retention, its plasma concentration is 10-fold higher than that of insulin with a biological half-life of more than 30-min in adult humans, compared to 3-4 min for insulin [3].

C-peptide was for long time considered to only possess the biological activity of favoring pro-insulin folding within the beta cells. Since it is secreted in the peripheral circulation in equimolar amount with insulin [4], the determination of C-peptide in serum and urine has been extensively used as a marker of endogenous insulin secretion to monitor the metabolic state of Type 1 and Type 2 diabetic patients. Similarly, measurement of circulating levels of C-peptide is considered the gold standard for islet/pancreas transplant function. In the last decade, interest in the possibility of a physiological effect of C-peptide originated from the observation that T1D patients who retain low, but detectable levels of C-peptide are less susceptible to develop diabetes-associated microvascular complications of the eyes, kidneys, and the peripheral nerves as compared to T1D patients in whom beta cell function ceases totally [5, 6]. Moreover, T1D patients receiving whole pancreas or allogenetic islet transplantation, with restoration of both insulin and C-peptide secretion, exhibited improvement in diabetes-induced endothelial dysfunction, and in nerve and kidney functional and structural changes, in comparison to patients receiving daily insulin injections to control their hyperglycemia [7-10]. A low frequency of chronic complications has recently been observed also in T1D patients transplanted with neonatal pig islets [11]. These beneficial effects have been attributed, at least in part, to the presence of C-peptide. It was therefore hypothesized that C-peptide represents an important factor in reversing or preventing the microvascular damage associated with diabetes. This view was recently supported by a study involving a large cohort of T1D patients in which presence of a remaining serum C-peptide level above 0.06 nM conferred a statistically significant protective effect against the development of microvascular complications independently of glycemic control, duration of diabetes, age and sex [12].

Research during the last years has provided direct and robust evidence that C-peptide is in effect capable of insulin-independent biological effects in many different cell types,

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C-peptide fulfills an important function in the assembly of the two-chains insulin structure

C-peptide is cleaved from proinsulin and released in the bloodstream in amounts equimolar to those of insulin

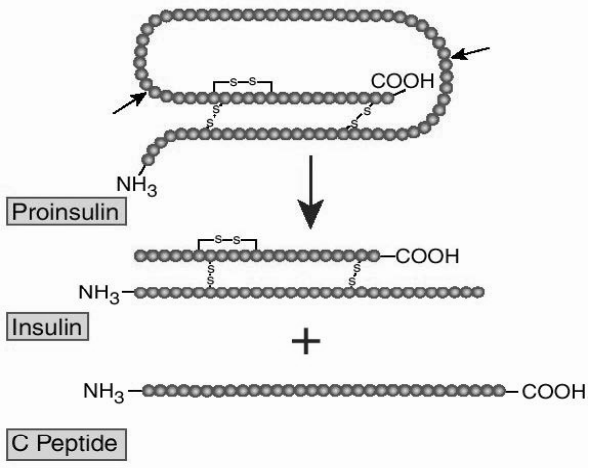

Fig. (1). Proinsulin C-peptide molecule. C-peptide is the peptide segment connecting the insulin A and B chains, and a product of proinsulin cleavage in the secretory granules, generated in the pancreatic beta-cells as part of normal insulin production. C-peptide is then secreted into the bloodstream in equimolar amount with insulin in response to elevated blood glucose levels. In this Figure is shown a schematic view of the proinsulin molecule. The black arrows indicate sites of cleavage by proteases at the level of Arginine residues. Once secreted into the bloodstream, C-peptide circulates at low nanomolar concentrations in healthy individuals, but it is absent in most patients with T1D.

where it affects activation of several intracellular pathways, such as, but not limited to, those involved in cellular proliferation and inflammation [13-15]. Importantly, results from small clinical trials have demonstrated that C-peptide is beneficial when administered as replacement therapy to T1D patients who suffers from diabetic vascular complications, in particular in relation to nephropathy, neuropathy, and augmentation of blood flow [16-19]. Currently, C-peptide replacement therapy is in Phase II clinical development by Cebix (which acquired Creative Peptides) for a diabetic neuropathy indication. In this review, we aim to present recent molecular studies showing that C-peptide has anti-inflammatory properties that could explain the beneficial effects of C-peptide on the vasculature of T1D patients. In addition, binding of C-peptide to cellular membranes and internalization to subcellular organelles will be also presented. Finally, intracellular signaling pathways affected by C-peptide in inflammatory responses will also be discussed.

INFLAMMATION IS A MAJOR COMPONENT OF T1D

T1D is a chronic disease characterized by disruption of glucose homeostasis and by concomitant metabolic abnormalities as the result of insulin deficiency secondary to progressive autoimmune destruction of the insulin-producing pancreatic beta-cells [20]. The adjacent glucagon (alpha)- and somatostatin (delta)-producing cells are not affected. Hyperglycemia, glycosuria, polydipsia, and weight loss are some of the most significant early clinical signs and symptoms of the diabetic syndrome that arises when a major proportion of the beta-cells are destroyed. During the course of diabetes, most T1D patients gradually develop one or several histopathological lesions affecting the vasculature of both large and small blood vessels, leading to macro- and microvascular complications [21]. These lesions result in the development of accelerated atherosclerosis, coronary artery disease, visual impairment, renal dysfunction and sensory loss, all of which seriously affect the quality and life style of diabetic patients [22-25].

T cells are recognized to play a central role in the autoimmune destruction of the insulin-producing beta-cells [26]. However, components of the innate immune system, such as natural killer cells and monocytes have a much broader role in the pathogenesis of T1D and associated vascular complications than previously recognized [27-30]. Monocytes are pivotal cells in inflammatory responses as they serve as the principal reservoir of pro-inflammatory cytokines and are the first cells to be engaged in nonspecific immune responses, such as those triggered by environmental factors. Studies in BB rats, an animal model of T1D, have shown that monocytes are the first elements to accumulate in the pancreatic islets in the early stages of the disease [31]. Upon secretion of inflammatory mediators by monocytes, subsequent T and B lymphocytes infiltration of the islets occurs [32], suggesting that monocytes and secreted inflammatory mediators contribute to the early induction and amplification of the autoimmune assault against the pancreatic beta-cells [33]. Activation of peripheral blood monocytes, with increased levels of biomarkers of inflammation and oxidative stress, has been found in adult T1D patients well after the onset of

diabetes as well as in children with recent-onset (<1 year) of T1D [34-39], suggesting presence of a systemic inflammatory response in T1D. In these T1D patients, monocytes release higher levels of the pro-inflammatory cytokines interleukin(IL)-1beta, IL-6 and IL-8, in both the resting state as well as after lipopolisaccharide (LPS) stimulation as compared to non-diabetic subjects. These findings demonstrate that a generalized inflammatory response is present in the very early stages of diabetes [40, 41]. Many of the reported inflammatory changes are detected at the level of monocytes of T1D patients, which show up-regulation of the adhesion molecule CD11b (Mac-1) [41] and have aberrant constitutive and LPS-stimulated expression of cyclooxygenase (COX)-2, a defect which may predispose to a chronic inflammatory response in T1D [30, 42].

The direct consequences of systemic monocyte activation in T1D are unknown, but theoretically could involve release of pro-inflammatory cytokines that could target the vascular endothelium of the pancreatic islets or the kidney and retina causing endothelial cell activation with increased monocyte-endothelium interactions, eventually leading to overt vascular damage in the later stages of T1D. Indeed, inflammation is now considered a major component in the development of T1D-associated vascular dysfunction [37, 43-45]. It is therefore important to understand the origin of the inflammatory response characterizing T1D, since therapeutic strategies to decrease inflammatory activity in T1D will likely improve endothelial function. In particular, it is difficult to decide to what extent the inflammatory state is a primary pathogenic event contributing to the development of T1D or if it is the response to a metabolic derangement, i.e., a situation of poor glycaemic control and insulin-deficiency present at the early stages of the disease [41, 46]. In the first case, the inflammatory state should precede overt diabetes and in this context the study of pre-diabetic subjects (i.e., autoantibody-positive individuals) should be characterized in respect to the inflammatory state.

T1D IS A RISK FACTOR FOR THE DEVELOPMENT OF ENDOTHELIAL DYSFUNCTION

Endothelial cells form a continuous dynamic layer of the blood vascular system that operates to deliver blood-borne nutrients and necessary levels of oxygen into tissues and, at the same time, to remove waste [47]. The endothelial cell lining is continuously exposed to a variety of biomechanical forces and stimuli and is thereby designated to be the homeostatic organ for the regulation of vascular tone and structure [48]. In addition, the endothelium has a regulatory role on leukocyte-endothelium interactions, leukocyte extravasation and subendothelial accumulation, all important events in the inflammatory cascade underlying vascular damage [49].

Chronic exposure of endothelial cells to insults and stressors shifts the normal endothelial function to a pathological degree, called endothelial dysfunction [49]. Endothelial dysfunction refers to a condition in which the ability of the endothelium to properly maintain vascular homeostasis is impaired [50]. Several pathological conditions have been identified as potential risk factors for the development of endothelial dysfunction, such as hypertension [51], chronic renal failure [52], Type 1 and Type 2 diabetes [53, 54], sedentary life style [55], and smoking [56]. These observations suggest that the pathophysiology of endothelial dysfunction is complex and involves multiple mechanisms.

Diabetes is a well-established risk factor for vascular diseases. Patients with T1D exhibit an increased susceptibility to develop a wide range of vascular complications, including microangiopathy and atherosclerosis, which account for the majority of deaths and disability in diabetic patients (Table **1**) [54]. Diabetes causes vascular compromise secondary to endothelial dysfunction. A major hallmark of diabetes is an abnormally elevated blood glucose level, i.e. hyperglycemia, which is one important factor causing endothelial dysfunction in diabetes. Acute (i.e., spike of hyperglycemia after a meal) and prolonged exposure of endothelial cells to high blood glucose are toxic to endothelial cells because they trigger massive changes in cellular phenotype, state of activation, metabolism and physiology. High glucose can exert a direct toxic effect on endothelial cells or through the generation of intermediate products such as advanced glycation end-products (AGE) [57, 58]. These changes are initially characterized by modification of endothelial cell function, such as by an increased vascular tone and permeability due to an impaired bioavailability of vasodilator factors such as nitric oxide and of vasoconstrictor factors such as endothelin

Table 1. Long-Term Complications of Type 1 Diabetes (T1D)

- **Retinopathy**

  Results in retinal edema, hemorrhage and loss of vision. The incidence of retinopathy increases over time. After 7 years of T1D, 50% of T1D patients have retinopathy. After 20 years of T1D, retinopathy affect 90% of diabetic patients.

- **Nephropathy**

  Results in microalbuminuria, proteinuria leading to gradual loss of kidney function. Develops in 35-45% of T1D patients.

- **Neuropathy**

  The most common form of diabetic neuropathy is a peripheral symmetric sensorimotor neuropathy developing within 5-10 years of T1D. Other forms include cranial and peripheral motor neuropathies and autonomic neuropathies affecting gastric and intestinal motility, erectile dysfunction, bladder function.

- **Cardiovascular disease**

  Coronary artery disease (CAD) is the main cause of death in T1D patients. By the age of 55, 35% of T1D patients die of coronary artery disease in comparison to 8% and 4% of nondiabetic men and women respectively

From Libby P *et al*. [21] and Nathan DM [23].

(ET)-1 [59]. High glucose exposure also modulates availability of permeability factors such as vascular endothelial growth factor (VEGF) [60]. A second sign of endothelial dysfunction is the modification of cell molecule expression on the surface of endothelial cells. These molecules are essential for leukocyte-endothelium interaction, the first step in atherosclerosis plaque formation. Generally, these adhesion molecules, such as vascular cell adhesion molecule(VCAM)-1, intercellular adhesion molecule(ICAM)-1, and selectins (L-selectine; or P-selectine), are expressed at low levels on endothelial cells and are up-regulated upon cellular activation, such as after exposure to high glucose or inflammation. Activated endothelial cells also increase secretion of certain pro-inflammatory cytokines, such as IL-6, IL-8 and monocyte chemoattractant protein (MCP)-1, which are pivotal in initiating leukocyte interactions with endothelial cells and their subsequent recruitment in the subendothelial layer (Fig. **2**) [61]. The migrated monocytes phagocyte oxidazed-low density lipoprotein(LDL), become foam cells and keep secreting inflammatory mediators that maintain inflammation by recruiting more immune cells from the bloodstream to the vessel wall. In the meantime, smooth muscle cells proliferate and migrate from the media to the intima of the vessel wall where the new atherosclerotic plaque is developing. IL-8 and MCP-1 are usually found in human atherosclerotic plaques [62]. Simultaneously, adipocyte cells are also recognized to secrete circulating cytokines (adipokines) such as IL-6, tumor necrosis factor(TNF)-α, leptin and plasminogen activator inhibitor(PAI-1), exacerbating the on-going vascular inflammation in the vessel wall of diabetic patients [63].

Additional players in the pathogenesis of hyperglycaemia-induced endothelial dysfunctions are reactive oxygen species (ROS). Hyperglycaemia increases oxidative stress and up-regulates the antioxidant enzyme machinery [64]. At some point, the antioxidant enzyme machinery is insufficient to combat oxidative stress. Furthermore, at later points in the disease, T1D patients actually have an impaired antioxidant response. It has been shown that hyperglycaemia-induced generation of ROS in endothelial cells occurs mainly through a NAD(P)H oxidase-dependent mechanism. NAD(P)H oxidase is an enzymatic complex made up by several subunits localized in the cytoplasm and plasma membrane. Specifically, NAD(P)H oxidase is composed by 2 membrane subunits ($p22^{phox}$ and $gp91^{phox}$) and 4 cytosolic subunits ($p40^{phox}$, $p47^{phox}$, $p67^{phox}$ and Rac-1). In a scenario of hyperglycaemia, NAD(P)H oxidase is stimulated and the cytosolic subunits translocate from the cytosol to the plasma membrane to induce ROS production [65]. Excessive ROS production is toxic to endothelial cells and leads to decreased cellular proliferation [66] and acceleration of the apoptotic process [67]. One of the mechanism(s) underlying ROS toxicity in the endothelium involves activation of transcription factors such as the nuclear factor(NF)-κB and activating protein(AP)-1 [57,68-70], which ultimately lead to activation of genes that increase production of inflammatory mediators and inflammatory responses in general. Increased oxidative stress is observed in peripheral blood monocyte from T1D patients suffering from microvascular complications compared to those without microvascular complications, as demonstrated by elevated levels of nitrotyrosine, monocyte superoxide anion [37], DNA and protein oxidation [71, 72]. From all these studies, it appears that inflammatory responses are important players in the development of endothelial dysfunction in patients with T1D.

ANTI-INFLAMMATORY ACTIVITY OF C-PEPTIDE PREVENTS ENDOTHELIAL DYSFUNCTION

It has been proposed that C-peptide exerts beneficial effects on the vasculature, especially when the vasculature is exposed to insults such as during T1D. Vascular disease in diabetes originates at the level of the endothelium with abnormalities in cellular function, a condition known as endothelial dysfunction, that precedes the development of structural abnormalities and damage. The healthy endothelium plays an important role in maintaining vessel wall homeostasis, synthesizing biologically active substances that modulate vascular tone and reactivity, preventing thrombosis, and influencing smooth muscle growth. In T1D some or all of these functions are altered. Studies during the last decades have started unraveling the molecular mechanisms underlying the vascular effects of C-peptide.

The adhesion and migration of circulating monocytes into the subendothelial space is one of the key events in the early stages of atherogenesis (Fig. **2**) [72, 73]. Surface molecules on both the leukocyte cells and the endothelium must be appropriately expressed to permit adhesion, binding and movement through the endothelial layer. Exposure to high glucose or other inflammatory stimuli triggers upregulation of cell adhesion molecules on endothelial cells [74-77], as well as release of chemotactic factors, including IL-8 and MCP-1 [57, 61] mediators which play a crucial role in leukocyte-endothelium interactions [61]. The first step in this process is mediated by selectins (L-selectin; or P-selectin) on the endothelium and carbohydrate moieties on the leukocytes. Tight binding of the leukocytes to the endothelium occurs through up-regulation of ICAM-1 and VCAM-1 on endothelial cells and activation of their specific counter-receptors on the leukocytes.

Several findings have shown that C-peptide affects leukocyte-endothelium interactions by reducing up-regulation of endothelial cell adhesion molecules typically observed under inflammatory conditions. The first evidence of this effect is from Scalia *et al.* [78], who demonstrated that pretreatment with C-peptide to rats injected with the inflammatory agents thrombin or N^G-nitro-L-L-arginine methyl ester (L-NAME), an agent causing acute endothelial dysfunction, resulted in reduced expression of ICAM-1 and P-selectin on the mesenteric microvascular endothelium. As a consequence, the number of rolling, adhering, and transmigrated leukocytes also decreased upon C-peptide administration to the animals. In another model of vascular injury, C-peptide decreased polymorphonuclear leukocyte (PMN) infiltration in isolated rat hearts following ischemia-reperfusion injury [79]. In this model, transendothelial migration of PMN to the subendothelial layers represents a prominent step in the inflammatory component of post-ischemic injury. PMN infiltration induces endothelial and myocardial injury by releasing cytotoxic substances such as oxygen derived free radicals, inflammatory cytokines and proteolytic enzymes. In neutrophil-depleted animals undergoing reperfused myocardial infarction, the infarct size was significantly decreased

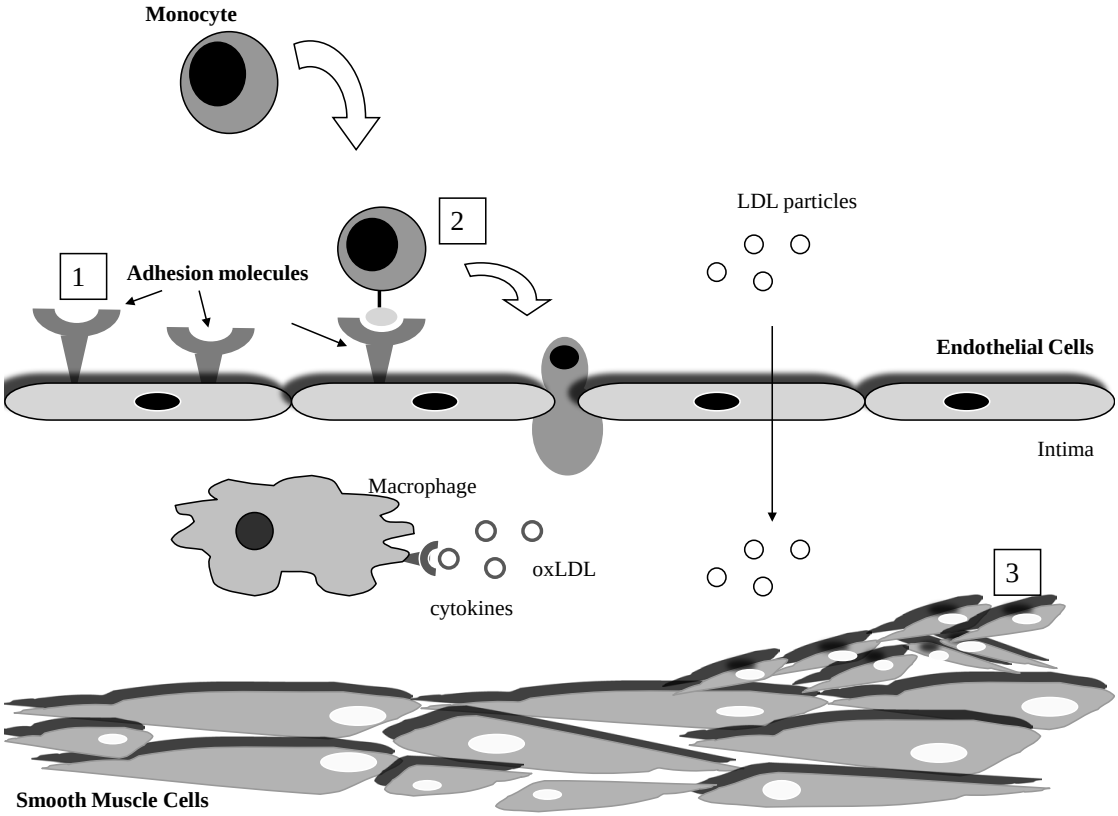

Fig. (2). Origin of vascular disease in diabetes. Exposure of endothelial cells to inflammatory insults, such as high glucose, causes inflammatory changes culminating in the up-regulation of cell surface adhesion molecules (i.e., VCAM-1), and secretion of pro-inflammatory cytokines (i.e., IL-8 and MCP-1) [1]. Circulating monocytes from diabetic patients change their phenotype in response to high blood glucose by expressing adhesion molecules (i.e., CD11b or Mac-1) that bind to their specific counter-receptors on activated vascular endothelial cells. As a consequence, monocytes and other circulating leukocytes roll and adhere to the activated endothelial cells [2]. Subsequently, monocytes migrate to the subendothelial space, where they phagocyte oxidized-LDL, secrete inflammatory chemokines that attract more immune cells in the subendothelial space. Macrophages become foam cells thus constituting the cellular core of the atherosclerotic plaque. Smooth muscle cells proliferate and migrate from the media to the intima of the vessel wall [3] where the new atherosclerotique plaque is developing.

demonstrating that a significant amount of myocardial injury is induced by neutrophil-associated mechanisms [79, 80]. In the study by Young *et al.*, by reducing PMN infiltration to the myocardium, systemic administration of C-peptide restored cardiac contractile function and postreperfusion coronary heart flow [79]. These findings have been recently recapitulated *in vitro* in a model of high glucose-endothelial dysfunction in which physiologic concentrations of C-peptide exerts an inhibitory effect on high glucose-induced up-regulation of the endothelial cell adhesion molecule VCAM-1 both at the mRNA and protein levels, and reduces by 50% the attachment of U-937 monocytes to endothelial cells as compared to condition in which C-peptide was not present in the medium [81]. These effects were observed as early as 4 hours after C-peptide addiction to the high glucose medium, and was still detected after 24 hour incubation. In the same model, C-peptide was also demonstrated to reduce high glucose-induced secretion of IL-8 and MCP-1 by human aortic endothelial cells (HAEC) to the basal levels measured under normal glucose concentrations [81]. These two chemokines are essential to promote leukocyte adhesion to endothelial cells. Conversely, when C-peptide was added to the medium containing normal glucose levels, it failed to significantly reduce VCAM-1 expression and IL-8 or MCP-1 secretion from HAEC, suggesting that the most meaningful biological effects of C-peptide on the endothelium are observable under conditions of vascular insult or damage.

ANTI-INFLAMMATORY ACTIVITY OF C-PEPTIDE IN ANIMAL MODELS OF SEPSIS

Sepsis, defined as acute systemic inflammatory response to infection, is the most common cause of death in intensive care units. It has been calculated that 10% of all deaths in the United States are attributable to sepsis [82]. The incidence is increasing and it is especially common in the elderly, thus it is likely to increase further as the population ages [82, 83]. Systemic inflammation is a pathogenetic component of severe sepsis and of other critical illness, such as in the context of major trauma, burns, pancreatitis, and after major surgery [84, 85]. A central feature of systemic inflammation is the perturbation of the immune system. This perturbation involves engagement of nonspecific immune mechanisms with both monocytes and granulocytes displaying activation-related changes in expression of surface antigens, increased production of ROS and excessive production of inflammatory cytokines. Cytokines are messengers for the regulation of the inflammatory cascade, with TNF-α, IL-1β, IL-6 and IL-8 working synergistically. Cytokines elicit a wide variety of biological activities on different cell populations, endothe-

lium included. On endothelial cells, pro-inflammatory cytokines can induce functional and structural alterations, including oxidative damage or interference with the mechanisms of contraction/relaxation leading to alterations in vascular integrity, tone, and coagulation, and lethal multiple organ failure [83, 84]. To date, the conventional therapies, combined with circulatory and respiratory support are not always adquate to reduce the systemic inflammation [85], and despite advances in medical knowledge, sepsis continues to carry a high morbidity and mortality rate. In this setting, anti-inflammatory strategies may be life-saving.

In a recent report, injections of C-peptide *in vivo* to LPS-treated mice, an animal model of endotoxic shock, drastically improved survival rate of the animals compared to the vehicle-treated mice. The beneficial effect of C-peptide in these LPS-treated animals was likely due to the fact that C-peptide reduced peripheral levels of the pro-inflammatory cytokines TNF-α and MCP-1 in LPS-treated mice as compared to vehicle-treated ones, leading to a decreased overall inflammatory response in the lung, an organ which is severely damaged in endotoxic shock [15]. C-peptide effect was mediated by increased DNA binding of the nuclear transcription factor peroxisome proliferator-activated-receptor (PPAR)-γ in the lung of endotoxin-treated mice, an event associated with inhibition of the phosphorylation of extracellular signal regulated kinases 1 and 2 (ERK1/2) in the lung [15], a pathway involved in the regulation of gene expression of several inflammatory mediators.

C-PEPTIDE AND COGNITIVE DYSFUNCTION IN T1D

Recent studies demonstrate the importance of C-peptide in modulating inflammatory pathways in the central nervous system. It is known that T1D patients may suffer impairments in learning, memory, problem solving, and mental and motor speed with primary diabetic encephalopathy recognized as a late complication of T1D [86]. In the type 1 BB/Wor rat, cognitive impairment is associated with apoptosis-induced neuronal loss in the hippocampus, an event associated with NF-κB and RAGE activation [97]. Elevation levels of the pro-inflammatory cytokines TNF-α, IL-1β, IL-2, and IL-6 was also present in these animals [89]. The upregulation of RAGE and activation of NF-κB, TNF-α, IL-1β, IL-2, and IL-6 were significantly reduced when the diabetic rats received C-peptide replacement which was also associated with the prevention of astrocyte proliferation and reduction of apoptosis [87, 88]. Other preliminary studies have also revealed deficits in temporal white matter with increased RAGE expression and pro-inflammatory cytokines which were significantly prevented by C-peptide replacement [88, 89].

INTRACELLULAR PATHWAYS MEDIATING THE ANTI-INFLAMMATORY EFFECTS OF C-PEPTIDE

The nuclear factor (NF)-κB pathway. The specific intracellular mechanisms underlying the anti-inflammatory effects of C-peptide on endothelial and other cells, are largerly unknown. Nevertheless, the major signal transduction pathways regulating inflammatory responses in a variety of cells require nuclear translocation of the transcription factor NF-κB [90]. In the unstimulated state, NF-κB exists as a heterodimer composed of p50 and p65 subunits bound to IκB in the cytoplasm. Upon activation, for example after cellular exposure to high glucose, IκB is phosphorylated and degraded, thus causing release of the p50/p65 components of NF-κB [91]. The active p50/p65 heterodimer translocates to the nucleus and initiates the transcription of a gamut of genes involved in the inflammatory response, such as pro-inflammatory cytokines, cell surface adhesion molecules, and chemokines, including IL-8 and MCP-1 [90-92]. In a recently published paper, it was demonstrated that in cultured HAEC physiological concentrations of C-peptide reduced nuclear translocation of the NF-κB p65 and p50 components that is normally observed under high glucose. In the same conditions, heat-inactivated C-peptide did not have any effects on nuclear translocation of NF-κB p65 and p50 subunits [81]. By reducing NF-κB nuclear translocation of p65/p50 subunits, C-peptide might reduce VCAM-1 expression as well as secretion of the inflammatory cytokines IL-8 and MCP-1 in HAEC exposed to high glucose and therefore decrease inflammation [81]. An effect of C-peptide on NF-kB and consequent decreased inflammatory cytokine production has also been reported in the brain of diabetic BB/Wor rats and found to be associated with reduced neuronal apoptosis [86-89].

The mechanism(s) by which C-peptide reduces NF-κB activation is not known. It is very likely that C-peptide acts on one NF-κB dependent upstream signaling events, such as IκB kinase activation, an enzyme that elicits phosphorylation of the cytosolic NF-κB inhibitor IκBα. This latter upstream event regulates NF-κB translocation from the cytoplasm to the nucleus. In vascular smooth muscle cells (VSMC), C-peptide reduces high glucose-induced proliferation [93,94]; a key event in atherogenesis, by reducing phosphorylation of IκBα, a pathway likely to be also targeted in endothelial cells. Another NF-κB dependent upstream event that needs to be elucidated as potential target for C-peptide effect is ROS generation. ROS are powerful cellular activators of the NF-kB pathway. C-peptide might reduce NF-κB activation by decreasing ROS generation in target cells, such as endothelial cells. In this case, one cellular target for C-peptide effect on decreasing ROS generation is the NAD(P)H oxidase enzyme, which is one pathway regulating ROS production in endothelial cells. C-peptide could either inhibit NAD(P)H oxidase assembly at the level of the plasma membrane by physically interacting with one or more subunits or by diminishing the activity of the assembled enzyme. Another possibility is that C-peptide directly interacts with NF-κB p65/p50 subunits at the nuclear level, preventing DNA binding. These are all fascinating possibilities that need to be investigated as mechanism(s) of action of C-peptide.

The peroxisome-proliferator-activated receptor γ (PPAR-γ) pathway. Another signaling pathway that has been reported to be affected by C-peptide is the one mediating activation of PPAR-γ [95], a member of the nuclear receptor superfamily of ligand-activated transcription factors. In addition to its function in adipogenesis, and increasing insulin sensitivity, PPAR-γ regulates the expression of several genes involved in inflammation and vascular disorders, such as atherosclerosis [96], by either controlling the gene transcrip-

tional machinery [97-99] or interacting with other transcription factors such as (AP)-1, (signal transducers and activators of transcription) STAT, and NF-κB [100]. Recently, it has been demonstrated that NF-κB is a major target of the anti-inflammatory activity of PPAR-γ in the endothelium, smooth muscle and macrophages [101]. PPAR-γ inhibits NF-κB activation by suppressing its promoter activity and/or by preventing phosphorylation of the NF-κB p65 subunit as well [102]. By neutralizing NF-κB activation, PPAR-γ modulates a constellation of inflammatory events crucial for the initiation of vascular diseases [103]. Activation of PPAR-γ by natural or synthetic ligands improves vascular dysfunction [104] and is emerging as a new therapeutic approach to treat diseases with a strong inflammatory component [15]. Although the effect of C-peptide on the activation of the PPAR-γ has not been investigated in the vasculature, there is evidence that C-peptide increases PPAR-γ phosphorylation in kidney proximal tubular cells, an effect mediated by activation of PI3K [95]. In the same study, C-peptide up-regulated the expression of the specific PPAR-γ-regulated gene CD36 [95], a scavenger receptor for oxidized-LDL uptake, in the monocytic cell line THP-1. PPAR-γ activation reduces the accumulation of atherogenic ox-LDL in the vascular wall by enhancing both uptake into and efflux out from macrophages [104]. A similar effect on PPAR-γ has been reported in lung epithelial cells of LPS-treated mice, an animal model of endotoxic shock [15]. Whether C-peptide extends its anti-inflammatory activity also on activated monocytes has not been investigated to date.

CELL BIOLOGY OF C-PEPTIDE INTERNALIZATION AND POSSIBLE MECHANISM OF ACTION

Although there is a general consensus that C-peptide has physiological effects on several different cell types, the cell biology of C-peptide is for the most part unknown. In particular, it is not known how exactly C-peptides exerts intracellular activities in target cells. It was initially thought that C-peptide exerted its effect *via* nonchiral mechanisms rather than by binding to stereospecific receptors [105]; though specific binding to cultured rat pancreatic beta cells was demonstrated earlier [106]. More recently, specific binding of C-peptide to cellular membranes has been confirmed in several human cell types including human renal tubular cells, human fibroblast and saphenous vein endothelial cells [107, 108]. Furthermore, C-peptide binding reaches full saturation at 0.9 nM; thus in healthy subjects, receptor saturation is achieved already at physiologic levels [108]. A specific C-peptide receptor has not been identified yet, although it is often suggested to be a G-protein-coupled receptor as deduced from effects of pertussis toxin [108, 109].

More recently, C-peptide was shown to cross plasma membranes localizing in the cytoplasm of HEK-293 cells and Swiss 3T3 fibroblasts [109], where it was detected up to 1 h after uptake of the peptide. A nuclear localization of C-peptide in HEK-293 cells and Swiss 3T3 fibroblasts has also been demonstrated by the same group [109]. These findings demonstrate that once internalized in the cytoplasm, C-peptide is not rapidly degraded but remains intact, possibly interacting with sub-cellular components through which it might achieve its cellular effects. Recently, C-peptide was detected in the nucleoli where it promoted transcription of genes encoding for ribosomal RNA [110]. Luppi *et al*. investigated the process of internalization from the cell surface and sub-cellular localization of C-peptide in target cells [111]. In particular it was investigated whether C-peptide passively diffuses across the cellular membrane or whether it is actively translocated by a specific pathway of internalization, such as endocytosis. To this purpose, Luppi *et al*. customized AlexaFluor-labeled C-peptide probes and used them to study C-peptide uptake in HAEC and umbilical artery smooth muscle cells (UASMC), two relevant targets of C-peptide activity, by employing confocal-laser scanning microscopy in a live-cell setting. It was found that C-peptide binds to plasma membranes and internalizes in the cytoplasm of both cell types. The uptake of C-peptide was minimal after 5 min of incubation at 37ºC with AlexaFluor488-labeled C-peptide probe and began to be clearly visible after 10 min. As a control for specificity of the staining, cells were incubated with the AlexaFluor488 fluorescent dye alone and this incubation resulted in the absence of staining [111]. Further, Luppi *et al*. tested whether C-peptide was directly translocated across the plasma membrane or whether it followed a vesicle-mediated pathway during internalization. In this latter case, the fate of C-peptide upon internalization from the cellular membrane might be to localize in classic endocytic organelles, such as endosomes. To explore this possibility, HAEC and UASMC were transduced with Organelle Lights™ Endosome-GFP reagent, which targets expression of fluorescent Rab5a, an early endosome-specific marker. Cells were then labeled with AlexaFluor546-labeled C-peptide probe at 37ºC for 30 min and imaged in a live-cell setting under a confocal microscope. In the majority of cases, it was found that the internalized C-peptide probe was evidently contained inside the endosome structures. C-peptide eventually trafficked to lysosomes in live HAEC and UASMC [111].

These findings indicate a process of C-peptide internalization from the cell surface within membrane-bound organelles of the endocytic pathway, and excluded direct translocation across the plasma membrane. Endosome localization of C-peptide would support the proposal that C-peptide might achieve its cellular effects in part by signaling from these organelles. Endosomes interact with a complex network of tubules and vesicles distributed throughout the cytoplasm interconnected by a tightly controlled transport system [112]. In addition to their classical role as sorting stations for internalized activated receptor-peptides complexes on their way to lysosomal degradation, endosomes are emerging as crucial players in intracellular signaling [113]. Examples of signaling endosomes are the ones associated with epidermal growth factor receptors (EGFRs) whose downstream signaling factors such as SHC-adaptor protein (SHC), growth factor receptor bund protein 2 (GRB2) and mammalian Son-of-sevenless (mSOS) were found not only on the plasma membrane but on early endosomes as well [114], suggesting that EGFR signaling continues in this compartment [115]. Another example of signaling endosomes is the one associated with nerve growth factor (NGF) that was found bound to its activated receptor TrkA and phospholipase C-γ1 (PLC-γ1) in endocytic organelles [116].

Based on the current findings, signaling from putative C-peptide/C-peptide-receptor complexes might initiate at the plasma membrane, continue from early endosomes, and terminate at the lysosomes. The effect of C-peptide on the NF-κB pathway might originate from C-peptide/C-peptide-receptor complex signaling from the endosomes, as it has been demonstrated for certain Toll-like receptor pathways and other inflammatory pathways, which affect activation of the NF-κB pathway from the endosomes [117, 118]. The mildly acidic pH of the sorting endosomes would then begin the dissociation of the C-peptide destined to lysosomes from its recycled receptor. C-peptide could inhibit the NF-κB pathway through activation of PPAR-γ (Fig. **3**), which in turn inhibits translocation of NF-κB p65/p50 components to the nucleus, thus reducing transcription of inflammatory genes. Another likely target for C-peptide is the enzyme NAD(P)H oxidase, the major ROS producer in endothelial cells (Fig. **3**). Future research has to determine the contributions of these mechanism(s) in C-peptide anti-inflammatory signaling.

CONCLUSIONS

T1D patients lack physiological levels of insulin and C-peptide in their bloodstream due to the autoimmune destruction of their pancreatic beta cells. T1D patients are also at increased risk to develop both micro- and macro-vascular complications. Unfortunately, the standard of care for T1D patients is solely insulin-replacement therapy. Despite the recently published evidence of the beneficial effect of C-peptide replacement therapy on diabetes-associated vascular complications, C-peptide is not prescribed. In this review we have presented the most updated findings showing that C-peptide has indeed anti-inflammatory activity on endothelial cells exposed to the damaging effect of glucose, a common condition in T1D. Although there is still much to learn about the cell biology and specific mechanism(s) of action of C-peptide, C-peptide is emerging as a new molecule with therapeutic potential in the treatment of diseases with a strong inflammatory component, such as sepsis, T1D and diabetes-associated vascular complications.

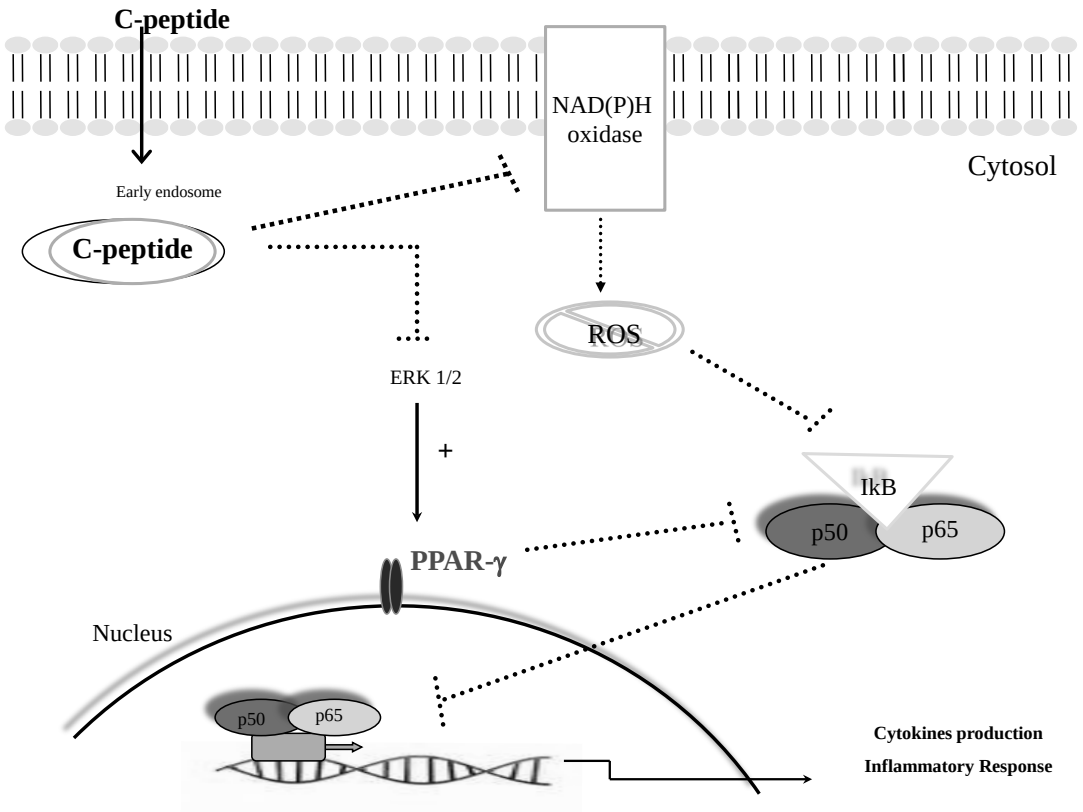

Fig. (3). Molecular mechanisms of anti-inflammatory activity of C-peptide. Based on the current findings, signaling from putative C-peptide/C-peptide-receptor complexes might initiate at the plasma membrane of target cells, continue from early endosomes, and terminate at the lysosomes. One of the major pathways involved in the anti-inflammatory effects of C-peptide in the vasculature is through the NF-κB pathway. How exactly C-peptide modulates the activity of this pathway is not clear yet, however several upstream signaling events along the NF-κB pathway have been recognized as likely intracellular targets for C-peptide. In this scenario, the inhibitory effect of C-peptide on the NF-κB pathway might originate from C-peptide/C-peptide-receptor complex signaling from the endosomes, as it has been demonstrated for certain Toll-like receptor pathways, which affect activation of the NF-κB pathway from these subellular organelles. C-peptide/C-peptide receptor complex could activate PPAR-γ, a nuclear receptor, either directly or *via* inhibition of phosphorylation of extracellular signal regulated-kinases(ERK)-1/2 which, in turn, activates PPAR-γ. PPAR-γ inhibits translocation of the NF-κB p65/p50 components to the nucleus, thus reducing transcription of inflammatory genes, such as those encoding for adhesion molecules and inflammatory cytokine secretion. Another likely target for C-peptide activity, is the enzyme NAD(P)H oxidase, the major producer of ROS in endothelial cells by preventing translocation of the cytoplasmic subunits to the plasma membrane, an event crucial for NAD(P)H oxidase activity. Future research in the field has to determine contributions of each of these mechanism(s) in C-peptide anti-inflammatory signaling. Dashed lines in the figure indicate inhibitory activity. Continued lines indicate stimulatory activity.

ABBREVIATIONS

ERK 1/2	=	Extracellular signal regulated kinases 1 and 2
HAEC	=	Human aortic endothelial cell
ICAM-1	=	Intercellular adhesion molecule-1
LPS	=	Lipopolysaccharide
MCP-1	=	Monocyte chemoattractant protein-1
NF-κB	=	Nuclear factor kappa B
PPAR-γ	=	Peroxisome proliferator-activated-receptor gamma
ROS	=	Reactive oxygen species
T1D	=	Type 1 diabetes
TNF-α	=	Tumor necrosis factor-alpha
UASMC	=	Umbilical artery smooth muscle cell
VCAM-1	=	Vascular cellular adhesion molecule-1

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ARTICLE

C-peptide reduces high-glucose-induced apoptosis of endothelial cells and decreases NAD(P)H-oxidase reactive oxygen species generation in human aortic endothelial cells

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Abstract
Aims/hypothesis Reactive oxygen species (ROS) generated during hyperglycaemia are implicated in the development of diabetic vascular complications. High glucose increases oxidative stress in endothelial cells and induces apoptosis. A major source of ROS in endothelial cells exposed to glucose is the NAD(P)H oxidase enzyme. Several studies demonstrated that C-peptide, the product of proinsulin cleavage within the pancreatic beta cells, displays anti-inflammatory effects in certain models of vascular dysfunction. However, the molecular mechanism underlying this effect is unclear. We hypothesised that C-peptide reduces glucose-induced ROS generation by decreasing NAD(P)H oxidase activation and prevents apoptosis
Methods Human aortic endothelial cells (HAEC) were exposed to 25 mmol/l glucose in the presence or absence of C-peptide and tested for protein quantity and activity of caspase-3 and other apoptosis markers by ELISA, TUNEL and immunoblotting. Intracellular ROS were measured by flow cytometry using the ROS sensitive dye chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2-DCDFA). NAD(P)H oxidase activation was assayed by lucigenin. Membrane and cytoplasmic levels of the NAD(P)H subunit ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1) (RAC-1) and its GTPase activity were studied by immunoblotting and ELISA. *RAC-1* (also known as *RAC1*) gene expression was investigated by quantitative real-time PCR.
Results C-peptide significantly decreased caspase-3 levels and activity and upregulated production of the anti-apoptotic factor B cell CLL/lymphoma 2 (BCL-2). Glucose-induced ROS production was quenched by C-peptide and this was associated with a decreased NAD(P)H oxidase activity and reduced RAC-1 membrane production and GTPase activity.
Conclusions/interpretation In glucose-exposed endothelial cells, C-peptide acts as an endogenous antioxidant molecule by reducing RAC-1 translocation to membrane and NAD(P)H oxidase activation. By preventing oxidative stress, C-peptide protects endothelial cells from glucose-induced apoptosis.

Keywords Apoptosis · Complications · C-peptide · Diabetes · Endothelial cells · Endothelial dysfunction · Inflammation · NAD(P)H · ROS · Vascular

Abbreviations

BAX	BCL-2-associated X protein
BCL-2	B cell CLL/lymphoma 2
CM-H_2-DCDFA	Chloromethyl-2′,7′-dichlorodihydro-fluorescein diacetate
DPI	Diphenyliodonium
HAEC	Human aortic endothelial cells
NF-κB	Nuclear factor of κ light polypeptide gene enhancer in B cells 1
RAC-1	Ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein RAC1)
ROS	Reactive oxygen species

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Introduction

Type 1 diabetes is a well-established risk factor for vascular disease [1]. Chronic elevations of blood glucose level (hyperglycaemia) and systemic low-grade inflammation contribute to the development of endothelial dysfunction, an early event in the pathogenesis of vascular disease in diabetes.

High glucose damages endothelial cells by increasing oxidative stress through generation of reactive oxygen species (ROS) [2–4], activation of the death protease caspase-3 [5], and inducing apoptosis [6, 7]. ROS are powerful cellular activators of the nuclear factor of κ light polypeptide gene enhancer in B cells 1 (NF-κB) pathway [8, 9], which regulates activation of a series of cytokine and adhesion molecule genes that results in the adhesion of leucocytes to endothelial cells and release of cytotoxic molecules. In human aortic endothelial cells (HAEC), activation of NF-κB accelerates apoptosis by downregulating production of B cell CLL/lymphoma 2 (BCL-2), an anti-apoptotic factor [10, 11].

High-glucose-induced ROS generation in endothelial cells mainly involves an NAD(P)H oxidase-dependent mechanism [12–15], which transfers electrons from NAD(P)H to molecular oxygen, producing O_2^-. The NAD(P)H oxidase enzyme is composed of four functional components, the assembly of which requires the presence of the small GTP-binding protein ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein RAC1) (RAC-1) at the plasma membrane [16, 17]. In endothelial cells, RAC-1 controls low-intensity basal superoxide production as well as bursts of NAD(P)H oxidase activity [16], such as during exposure to high glucose [18, 19]. In type 1 diabetes, RAC-1-mediated ROS generation is considered an important pathophysiological pathway in the development of vascular complications [14, 20, 21]. In a recent report, it was shown that glucose-induced NAD(P)H oxidase activation, inflammatory responses and cardiovascular complications were attenuated in an animal model of *Rac-1* (also known as *Rac1*) knockout [22]. This suggests that targeting inhibition of RAC-1 may represent an attractive therapeutic approach for reducing inflammatory-induced vascular damage in diabetes.

C-peptide, the cleavage product of the proinsulin molecule in the pancreatic beta cells, has been shown to exert insulin-independent biological effects on a number of cells, proving itself as a bioactive peptide with anti-inflammatory properties [23]. As type 1 diabetes patients typically lack physiological levels of insulin and C-peptide, this is considered an important factor in the pathophysiology of diabetic complications [24–26]. C-peptide has been shown to improve endothelial dysfunction and systemic inflammation in several in vivo and in vitro models of inflammation-mediated vascular injury by reducing expression of genes encoding endothelial cell adhesion molecules, inflammatory cytokine production and adherence and transmigration of leucocytes [27–30]. Although the exact mechanism(s) underlying the anti-inflammatory activity of C-peptide is not known, there is evidence that C-peptide affects NF-κB activation [29, 31]. However, which NF-κB-dependent upstream signalling event is affected by C-peptide in endothelial cells is not clear.

We hypothesised that C-peptide acts as an antioxidant molecule by reducing high-glucose-induced ROS generation in endothelial cells. Therefore, in this study, we examined the effect of C-peptide on high-glucose-induced ROS generation as the mechanism underlying its beneficial effects on endothelial cell dysfunction and apoptosis. We focused on the effect of C-peptide on the RAC-1 pathway of ROS generation, which is recognised as the major pathway of ROS production in endothelial cells during diabetes.

Methods

Cells HAEC were obtained from Lonza (Lonza, Walkersville, MD, USA) and maintained in T-75 cm^2 flasks (Corning, New York, NY, USA) at 37°C, 95% air and 5% CO_2 in EBM-2 (Lonza) supplemented with endothelial growth medium 2 (EGM-2) kit SingleQuots (Lonza). EBM-2 contains 5.5 mmol/l glucose, which is considered the normal glucose level required for HAEC survival. In all experiments, the high-glucose medium was EBM-2 containing 25 mmol/l glucose (Sigma-Aldrich, St Louis, MO, USA). HAEC were used when they reached 90% confluency and up to the sixth passage.

Treatment conditions HAEC were exposed to regular EBM-2, high-glucose medium, or high-glucose medium with either human C-peptide (Phoenix Pharmaceuticals, Burlingame, CA, USA) or scrambled human C-peptide (Sigma-Genosys, The Woodlands, TX, USA) (10 nmol/l) (purity ≥95%) for a time period ranging from 30 min to 48 h, as specified in each experiment. In experiments to detect *RAC-1* mRNA and protein production, and RAC-1 GTPase and NAD(P)H oxidase activities, human EGF was removed from the media to avoid aspecific RAC activation. To study TNF-α-mediated apoptosis, HAEC were pre-treated for 24 h with C-peptide (10 nmol/l) and then exposed to TNF-α (20 ng/ml) (R&D Systems, Minneapolis, MN, USA) for 24 h. All experiments were performed at 37°C, 95% air and 5% CO_2. The dose of 10 nmol/l C-peptide was selected because it showed significant anti-apoptotic effects in dose–response experiments. Unless otherwise indicated,

for each assay a minimum of three independent experiments were run in which each condition was tested in triplicate.

Detection of apoptosis HAEC were seeded in 96 well plates and the next day treated as specified above for 48 h. Apoptosis was detected using the Cell Death Detection ELISA^{PLUS} kit (Roche Diagnostics, Mannheim, Germany). Results were expressed as absorbance raw data (mean±SD). Apoptosis was also detected with a TUNEL assay using the In Situ Cell Death Detection Kit, Fluorescein (Roche Diagnostics) according to the manufacturer's instructions. This assay was performed on HAEC seeded on MatTek plates (MatTek, Ashland, MA, USA) and exposed for 96 h to the treatment conditions as above. The label incorporated at the damaged sites of DNA was visualised by confocal fluorescent microscopy (Olympus Fluoview PV1000, Center Valley, PA, USA) at ×40 magnification.

Immunoblotting for BAX, BCL-2, cleaved caspase-3 and RAC-1 For BCL2-associated X protein (BAX), BCL-2 and cleaved caspase-3 protein detection, HAEC were exposed overnight to the treatment conditions as above. For RAC-1 detection, HAEC were serum starved overnight before exposing to the treatment conditions for 30 min. Cytosolic and membrane proteins were extracted using Qproteame Cell Compartment kit (Qiagen, Valencia, CA, USA) and protein content was measured using a bicinchoninic acid assay kit (Pierce Biotechnology, Thermo Scientific, Rockford, IL, USA). Aliquots of protein extracts (30 μg) were subject to immunoblot analysis using rabbit polyclonal anti-RAC-1 (1:1000), anti-cleaved caspase-3 (1:500), anti-BCL-2 antibodies (all from Cell Signaling Technology, Danvers, MA, USA) and mouse monoclonal anti-β-actin antibody (1:10,000; Sigma). A rabbit polyclonal antibody anti-BAX (1:500) (Millipore, Billerica, MA, USA) was used to detect BAX protein levels. Densitometry was performed with UN-SCAN-IT gel software (Silk Scientific, Orem, UT, USA).

Assays of caspase-3 enzyme activity HAEC were maintained in 96 well plates and exposed to treatment conditions as above overnight. Caspase-3 activity was assessed in cytoplasmic cell lysates using the Caspase-3 Activity Assay Kit following manufacturer's instructions (Calbiochem, EMD Chemicals, Gibbstown, NJ, USA). Results were expressed as caspase-3 activity fold induction vs normal glucose condition (mean±SD).

Determination of intracellular ROS HAEC (50,000/well) were seeded in six-well plates and treated overnight as specified above. Intracellular hydrogen peroxide (H_2O_2) production was monitored over time by flow cytometry using chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2-DCFDA; 10μmol/l; Molecular Probes, Invitrogen), as specified in the electronic supplementary material (ESM). At least four experiments were run in which each condition was tested in duplicate. Results are expressed as mean fluorescence of CM-H_2-DCFDA.

NAD(P)H oxidase activity detection Glucose-induced NAD(P)H oxidase activity was measured in live HAEC exposed to the different treatment conditions for 30 min using lucigenin-derived chemiluminescence, as described by Mustapha et al. [32]. For a more detailed description of methods, see the ESM. Three experiments were performed in which each condition was tested in quadruplicate. Results were expressed as percentage (mean±SD) of NAD(P)H oxidase activity.

Measure of RAC-1 mRNA expression by quantitative real-time PCR HAEC were serum starved overnight and exposed to treatment conditions for 30 min. Total RNA was isolated using RNAqueous-4PCR kit (Ambion, Austin, TX, USA) and quantified by spectrophotometry. RNA, 1 μg, was reverse transcribed to cDNA (5 min at 65°C, 50 min at 50°C and 5 min at 85°C) using oligo(dT) primers (Invitrogen, Carlsbad, CA, USA) and quantitative real-time PCR was performed to amplify *RAC-1* and the housekeeping gene human *GAPDH* [33]. Sequences of the oligonucleotides used to amplify these genes are reported in the ESM. *RAC-1* data were normalised using the *GAPDH* housekeeping gene and results were expressed as fold induction vs normal glucose conditions (mean±SD of three independent experiments).

Assessment of RAC-1 GTPase activity HAEC were serum starved overnight and exposed to treatment conditions for 30 min. RAC GTPase activity was measured in 10 μg of cell lysates using the RAC G-LISA Activation Assay kit following the manufacturer's instructions (Cytoskeleton, Denver, CO, USA). At least four experiments were run in which each condition was tested in duplicate. Results are expressed as fold induction of GTPase activity (mean±SD) compared with normal glucose conditions.

Statistical analysis ANOVA followed by Dunnett's post hoc test was used to assess differences between the different conditions using GraphPad Prism 4 (GraphPad Software, San Diego, CA, USA). Values of $p<0.05$ were considered to be statistically significant.

Results

C-peptide decreases high-glucose-induced apoptosis of HAEC Exposure of HAEC to high glucose for 48 h significantly increased apoptosis as compared with normal

glucose (Fig. 1a,b; $p<0.01$). The addition of 10 nmol/l C-peptide decreased glucose-induced apoptosis of HAEC (Fig. 1a,b; $p<0.01$ vs high glucose). This effect corresponded to a 25% reduction of apoptosis by C-peptide as compared with high glucose. Higher concentrations of C-peptide (20 and 50 nmol/l) did not have any significant effects on glucose-induced apoptosis (Fig. 1a). In Fig. 1b, addition of scrambled C-peptide (10 nmol/l) to the high-glucose medium did not cause any significant effects on HAEC apoptosis, suggesting that the beneficial effect was specific to C-peptide.

Glucose-induced endothelial apoptosis was also evaluated by TUNEL assay under a confocal fluorescent microscopy (Fig. 2). As compared with normal glucose, HAEC exposed to high glucose demonstrated a significant induction of apoptosis that was reduced by C-peptide (10 nmol/l). Scrambled C-peptide was without any significant effect as compared with high glucose alone (Fig. 2).

C-peptide decreases high-glucose-induced caspase-3 production and activity in HAEC One crucial mediator of apoptosis is the activated caspase-3 protease, which catalyses the cleavage of many key cellular proteins. We evaluated endogenous levels of the large fragment

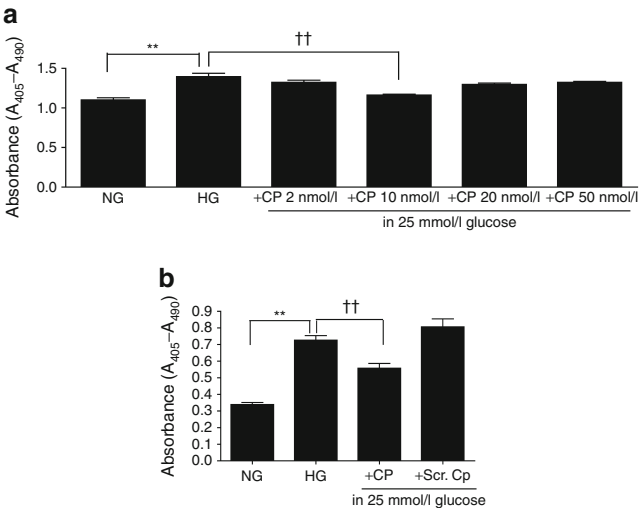

Fig. 1 C-peptide decreases glucose-induced apoptosis of HAEC. **a** HAEC were exposed to normal glucose, or to high glucose (25 mmol/l) alone or in the presence of a range of C-peptide concentrations for 48 h and tested for cytoplasmic histone-associated DNA fragments by using the Cell Death Detection ELISA^{PLUS}. **b** HAEC were exposed to normal glucose, or to high glucose alone or with either C-peptide or scrambled C-peptide (10 nmol/l) for 48 h and tested for apoptosis as in (**a**). A significant increase in apoptosis was found in high-glucose-exposed HAEC compared with cells exposed to normal glucose (**$p<0.01$). C-peptide at 10 nmol/l, but not scrambled C-peptide, decreased apoptosis ($^{\dagger\dagger}p<0.01$ vs high glucose). Higher concentrations of CP were not effective. Values are mean±SD of three different experiments in which each condition was tested in triplicate. CP, C-peptide; HG, high glucose; NG, normal glucose; Scr., scrambled

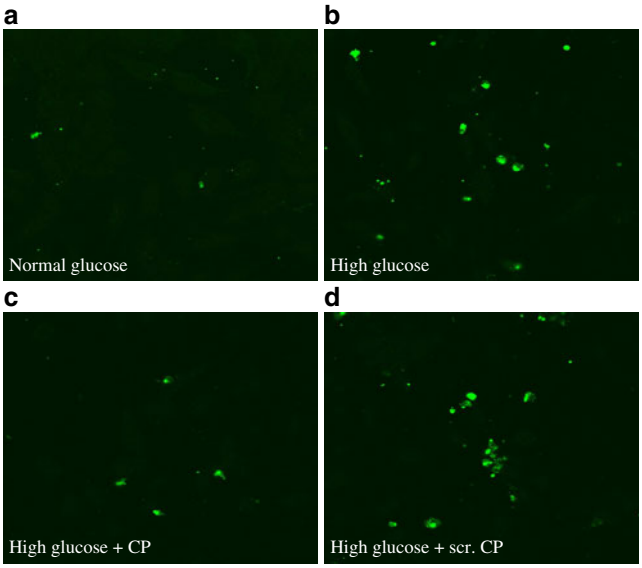

Fig. 2 TUNEL assay in glucose-exposed HAEC cultures: (**a**) normal glucose; (**b**) high glucose; (**c**) high glucose+C-peptide; and (**d**) high glucose+scrambled C-peptide. TUNEL staining shows an increase in apoptosis in HAEC (in green) exposed to high glucose compared with cells exposed to normal glucose. C-peptide reduced the number of TUNEL^{+} cells compared with high glucose alone. Scrambled C-peptide had no apparent effect. Shown are representative images of three independent experiments (×40 magnification). CP, C-peptide; scr., scrambled

(17/19 kDa) of activated (cleaved) caspase-3 by western blotting in cytoplasmic lysates from HAEC exposed to high glucose overnight. As shown in Fig. 3a, production of activated caspase-3 doubled in lysates from HAEC exposed to high glucose compared with normal glucose ($p<0.05$). Addition of C-peptide reduced caspase-3 levels to those detected in normal glucose ($p<0.05$ vs high glucose), a result that was not observed with scrambled C-peptide (Fig. 3a).

Caspase-3 activity was evaluated in cytoplasmic lysates from high-glucose-exposed HAEC by ELISA. Exposure to high glucose overnight significantly increased caspase-3 activity 1.5-fold compared with normal glucose (Fig. 3b; $p<0.01$). Addition of C-peptide, significantly reduced caspase-3 activity to levels detected in normal glucose ($p<0.01$ vs high glucose alone), while scrambled C-peptide showed no significant effects (Fig. 3b).

C-peptide increases production of the anti-apoptotic factor BCL-2 in high glucose-treated HAEC Analysis of the product of the survival gene *BCL-2* by western blotting showed that overnight exposure to high glucose decreased BCL-2 production by 50% compared with levels detected in normal glucose (Fig. 4a, $p<0.05$). Addition of C-peptide increased BCL-2 production to levels detected under normal glucose (Fig. 4a, $p<0.05$ vs high glucose). C-peptide did not change the levels of the pro-apoptotic

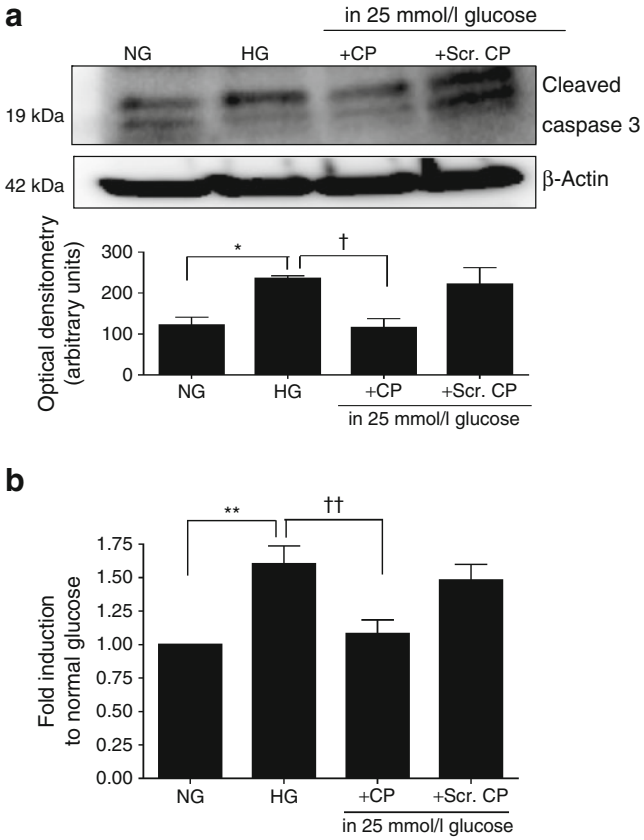

Fig. 3 C-peptide decreases cleaved caspase-3 protein levels and activity in HAEC exposed to high glucose. HAEC were cultured in normal glucose or high glucose (25 mmol/l) in the presence or absence of either C-peptide or scrambled C-peptide (10 nmol/l) overnight. **a** Cytoplasmic extracts were subjected to western blotting to detect cleaved caspase-3. Densitometric quantification of the bands showed that in cells exposed to high glucose there was a twofold increase in caspase-3 protein levels compared with cells exposed to normal glucose (*$p<0.05$). C-peptide significantly decreased caspase-3 levels ($^{\dagger}p<0.05$ vs high glucose). **b** A 1.5-fold increase in caspase-3 activity was measured in high-glucose-exposed HAEC compared with those exposed to normal glucose (**$p<0.01$). C-peptide treatment overnight reduced caspase-3 activity to levels detected in normal glucose ($^{\dagger\dagger}p<0.01$ vs high glucose). Results are expressed as mean±SD ($n=3$). CP, C-peptide; HG, high glucose; NG, normal glucose; Scr., scrambled

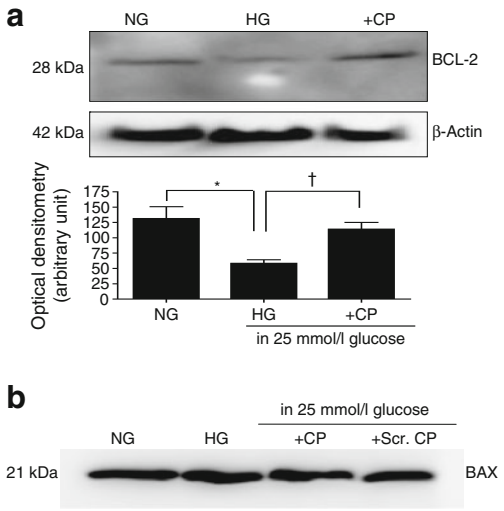

Fig. 4 C-peptide increases BCL-2 production in HAEC exposed to high glucose. **a** Representative immunoblot of BCL-2 and β-actin in extracts from HAEC incubated overnight in: normal glucose; high glucose (25 mmol/l); or high glucose+10 nmol/l C-peptide. Densitometric quantification of the bands showed significantly lower BCL-2 levels in high-glucose-exposed cells compared with those exposed to normal glucose (*$p<0.05$). Addition of C-peptide triggered an increase in BCL-2 levels ($^{\dagger}p<0.05$ vs high glucose). Results are expressed as mean±SD ($n=3$). **b** Representative immunoblot of the pro-apoptotic molecule BAX in extracts from HAEC incubated overnight in: normal glucose; high glucose (25 mmol/l); or high glucose+C-peptide or scrambled C-peptide (10 nmol/l). C-peptide did not change the production of BAX in glucose-exposed HAEC. CP, C-peptide; HG, high glucose; NG, normal glucose; Scr., scrambled

molecule BAX in glucose-exposed HAEC compared with cells exposed to high glucose (Fig. 4b).

C-peptide reduces TNF-α-mediated apoptosis of HAEC As an additional model of apoptosis, we investigated the one mediated by the inflammatory cytokine TNF-α, which plays an important role in the development of diabetic complications [34]. Exposure of HAEC to TNF-α significantly increased apoptosis as compared with normal glucose (Fig. 5a; $p<0.01$). Addition of C-peptide significantly reduced apoptosis as compared with TNF-α alone (Fig. 5a; $p<0.01$).

Caspase-3 levels were higher in lysates from HAEC treated with TNF-α compared with normal glucose (Fig. 5b). C-peptide reduced activated caspase-3 to levels observed under normal glucose (Fig. 5b). Analysis of BCL-2 production by western blotting showed that while TNF-α decreased BCL-2 levels compared with normal glucose, addition of C-peptide reversed this condition by increasing BCL-2 production to levels detected under normal glucose (Fig. 5b).

C-peptide reduces high glucose-induced ROS production in HAEC Figure 6a shows results from a representative experiment in which intracellular ROS generation in HAEC was assessed over time by flow cytometry analysis of the ROS sensitive dye CM-H_2-DCFDA in the gated cells. We found that at time 0, after overnight incubation with normal glucose (blue), HAEC showed a basal level of ROS, which continued to increase over time up to 3 h. ROS production was higher in HAEC exposed to high glucose (red) ($p<0.05$). In HAEC treated with high glucose and C-peptide (green), ROS generation was always lower than in cells exposed to high glucose alone (red) and this difference reached statistical significance at

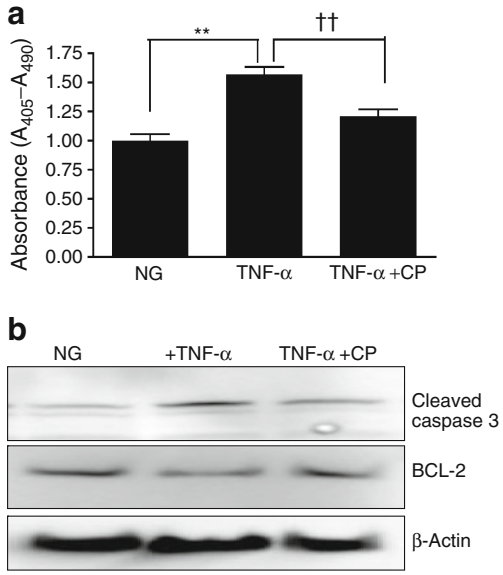

Fig. 5 C-peptide antagonises TNF-α-mediated apoptosis of HAEC. HAEC were exposed to normal glucose with or without TNF-α (20 ng/ml) in the presence or absence of C-peptide (10 nmol/l) for 24 h. **a** Changes in cytoplasmic histone-associated DNA fragments detected using the Cell Death Detection ELISA^{PLUS}. A significant increase in apoptosis was observed in TNF-α-exposed HAEC compared with those exposed to normal glucose alone (**$p<0.01$). Addition of C-peptide significantly reduced TNF-α-induced apoptosis as compared with HAEC exposed to TNF-α alone ($^{\dagger\dagger}p<0.01$). Results are expressed as mean±SD ($n=3$). **b** Representative image of immunoblot showing cleaved caspase-3 levels in HAEC exposed to the different conditions as above. While endogenous cleaved caspase-3 levels increased after exposure to TNF-α compared with medium alone, addition of C-peptide decreased caspase-3 levels. BCL-2 protein levels in HAEC decreased after exposure to TNF-α. Addition of C-peptide increased BCL-2 production to levels detected with normal glucose

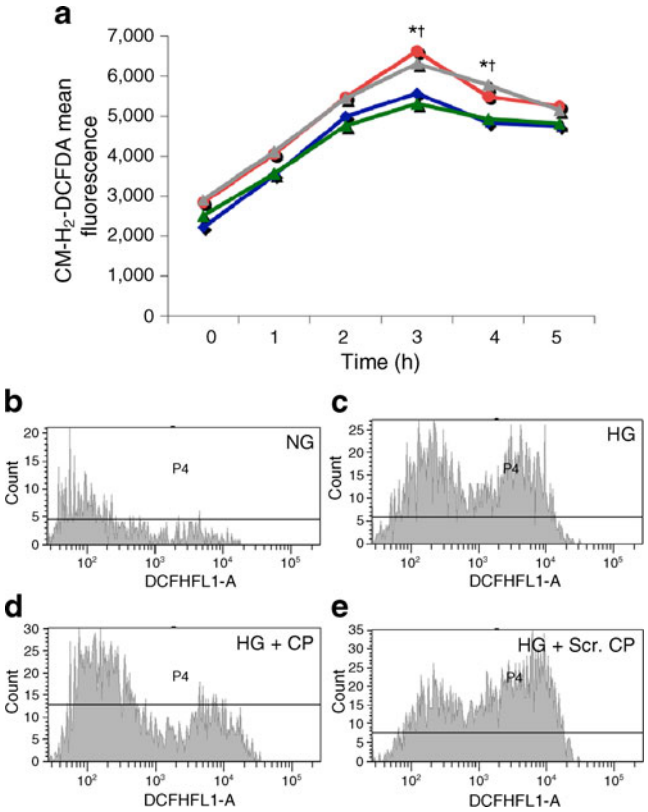

Fig. 6 C-peptide reduces high-glucose-induced ROS generation in HAEC. Intracellular ROS accumulation in HAEC exposed overnight to normal glucose (blue); high glucose (25 mmol/l; red); high glucose+C-peptide (green) or high glucose+scrambled C-peptide (10 nmol/l; grey). The following day, the ROS-sensitive dye CM-H_2-DCFDA (10 μmol/l) was added for 30 min in an incubator after which cells were run on a flow cytometer (time 0) and every hour for a total of 5 h. **a** Representative time course analysis of ROS generation expressed as mean fluorescence intensity of CM-H_2-DCFDA. At time 0, after overnight incubation, HAEC in normal glucose showed a basal level of ROS, which continued to increase over time up to 3 h. HAEC in high glucose produced higher ROS (*$p<0.05$ vs normal glucose). C-peptide lowered glucose-induced ROS production at all time points, but reached significance at 3 h and 4 h ($^{\dagger}p<0.05$ vs high glucose). Scrambled C-peptide had no significant effect compared with high glucose alone. **b–e** Representative histograms of flow cytometry analysis of ROS detection at 3 h: (**b**) normal glucose; (**c**) high glucose; (**d**) high glucose +C-peptide; (**e**) high glucose+scrambled C-peptide. Each histogram shows fluorescence intensity (on the *x*-axis) and number of events (counts) on the *y*-axis. The peak on the left represents negative cells, while the peak on the right represents cells that positively stain with CM-H_2-DCFDA. In the histogram of cells exposed to high glucose (**c**), the number of CM-H_2-DCFDA positive cells increased as compared with normal glucose (**b**). When C-peptide was added to the high glucose medium (**d**), the number of CM-H_2-DCFDA-positive cells decreased, while scrambled C-peptide was without any significant effects (**e**)

the 3 h and 4 h time points ($p<0.05$) (Fig. 6a). When scrambled C-peptide was added to high glucose (grey), no significant decrease in DCFDA fluorescence was detected in HAEC as compared with cells exposed to high glucose alone (red) (Fig. 6a). Figure 6b shows representative histograms of flow cytometry analysis of CM-H_2-DCFDA fluorescence in HAEC under the different treatment conditions at the 3 h time point.

C-peptide reduces NAD(P)H oxidase activity in high glucose-exposed HAEC Exposure of HAEC to high glucose for 30 min increased NAD(P)H oxidase activity of 50% as compared with normal glucose ($p=0.01$). C-peptide added to the medium for 30 min significantly down-regulated NAD(P)H oxidase activity as compared with high glucose alone ($p<0.01$), while scrambled C-peptide did not have any significant effect (Fig. 7). As expected, the pharmacological NAD(P)H oxidase inhibitors diphenyliodonium (DPI) and apocynin significantly abolished high-glucose-induced NAD(P)H oxidase activation ($p<0.01$ and $p=0.01$, respectively).

C-peptide reduces high-glucose-induced production of RAC-1 at the plasma membrane and its GTPase activity Assembly of the four functional components to form active NAD(P)H

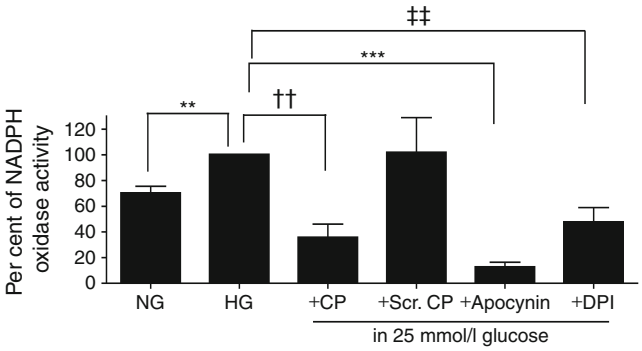

Fig. 7 C-peptide reduces high-glucose-induced NAD(P)H oxidase activation in HAEC. HAEC were exposed to: normal glucose; or high glucose (25 mmol/l) in the presence or absence of either C-peptide or scrambled C-peptide (10 nmol/l) as a control. After 30 min, high glucose increased NAD(P)H oxidase activity compared with normal glucose (**p=0.01). C-peptide, but not scrambled C-peptide, reduced high-glucose-induced NAD(P)H oxidase activity ($^{\dagger\dagger}p$<0.01 vs high glucose). Pre-treatment with the pharmacological inhibitors apocynin (10 μmol/l) and DPI (100 μmol/l) drastically reduced high-glucose-induced NAD(P)H oxidase activity in HAEC ($^{\ddagger\ddagger}p$=0.01 DPI vs high glucose; ***p<0.001 apocynin vs high glucose). Results are expressed as percentage (mean±SD) of NAD(P)H oxidase activity in three independent experiments. CP, C-peptide; HG, high glucose; NG, normal glucose; Scr., scrambled

oxidase requires the presence of the small GTP-binding protein RAC-1. Therefore, we investigated RAC-1 protein levels in the cytoplasm and plasma membrane of high-glucose-exposed HAEC by western blotting. Exposure of HAEC to high glucose for 30 min triggered translocation of RAC-1 from the cytoplasm to the plasma membrane as compared with exposure to regular medium (Fig. 8a; p<0.05). C-peptide significantly reduced RAC-1 translocation from the cytoplasm to the membrane (p<0.05 vs high glucose; Fig. 8a).

RAC is a member of the Rho family of small GTPases that undergo regulatory control by alternating between binding GTP for activation and hydrolysis to GDP for inactivation. We investigated whether intrinsic RAC-1 GTPase activity was affected by C-peptide. In Fig. 8b, HAEC exposed to high glucose for 30 min significantly increased GTPase activity by 50% compared with cells exposed to regular glucose medium (p<0.01). When C-peptide was added to high glucose, it decreased the GTPase activity of 25% compared with high glucose alone (p<0.01). Scrambled C-peptide did not significantly affect GTPase activation.

C-peptide does not affect RAC-1 mRNA gene expression in high-glucose-exposed HAEC We tested whether C-peptide treatment for 30 min had any effects on *RAC-1* mRNA gene expression in high-glucose-exposed HAEC. As shown in Fig. 8c, we did not find any significant differences in *RAC-1* mRNA expression in HAEC exposed to the different conditions tested.

Discussion

It has been reported that high glucose increases ROS generation in HAEC [2–4] and triggers apoptosis [6, 7, 10]. ROS production causes apoptotic cell death in endothelial cells [5, 7, 10] and plays an important role in the development of diabetic vascular complications [2, 4]. Indeed, it has been shown that antioxidant agents rescue hyperglycaemia-induced endothelial dysfunction and reduce the risk of coronary heart disease [35, 36]. In this study, we have demonstrated that C-peptide reduced high-glucose-induced apoptosis and quenched glucose-induced

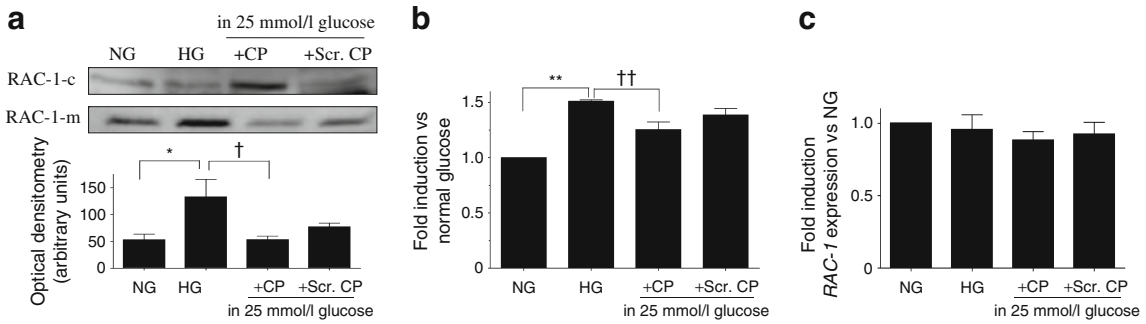

Fig. 8 Effect of C-peptide on RAC-1 translocation and GTPase activity in HAEC exposed to high glucose. HAEC were serum starved overnight and exposed to normal glucose or high glucose (25 mmol/l) in the presence or absence of either C-peptide or scrambled C-peptide (10 nmol/l) for 30 min at 37°C. **a** Western blot of cytoplasmic (RAC-1-c) and membrane (RAC-1-m) in glucose-exposed HAEC. Bar graphs show densitometric quantification of RAC-1-m. High glucose induced increased levels of RAC-1 in the plasma membrane (*p<0.05 compared with normal glucose). C-peptide treatment decreased translocation of RAC-1 from the cytoplasm to the membrane ($^{\dagger}p$<0.05 vs high glucose). **b** Cell lysates were subjected to the G-LISA assay to detect RAC GTPase activity. High glucose increased GTPase activity in HAEC after 30 min compared with normal glucose (**p<0.01). Addition of C-peptide decreased GTPase activity to levels measured in normal glucose ($^{\dagger\dagger}p$<0.01 vs high glucose). **c** Quantitative real-time PCR analysis of *RAC-1* mRNA gene expression in HAEC after 30 min exposure to the different treatment conditions as above. No significant differences were found in *RAC-1* mRNA gene expression in cells exposed to the various treatments. Results are expressed as (mean±SD) of three independent experiments

oxidative stress in endothelial cells, an effect conveyed through the inhibition of NAD(P)H oxidase. Furthermore, we demonstrated that the effect of C-peptide on glucose-induced NAD(P)H-oxidase-derived ROS production is mediated by an inhibition of RAC-1 translocation, a crucial component of NAD(P)H oxidase.

C-peptide is the cleavage product of the proinsulin molecule generated in the pancreatic beta cells of healthy individuals and co-released together with insulin in the peripheral circulation in response to elevation of blood glucose levels. In individuals with type 1 diabetes, both insulin and C-peptide are missing because of autoimmune destruction of the pancreatic beta cells. As a consequence, individuals with type 1 diabetes have severely reduced levels or absence of C-peptide; this is considered an important factor in the pathophysiology of diabetic complications. In fact, people with type 1 diabetes who retain a low but detectable level of C-peptide are less prone to develop microvascular complications of the eyes, kidneys and peripheral nerves [24–26]. Moreover, pancreas or islet transplantation, with restoration of endogenous insulin and C-peptide secretion, is known to be accompanied by improvement of diabetes-induced abnormalities of nerve function, endothelial function and both structural and functional changes of the kidneys [37, 38]. C-peptide has been shown to display anti-inflammatory activity on endothelial cells exposed to a variety of damaging insults and to be beneficial in endothelial dysfunction during type 1 diabetes [39]. In this regard, pretreatment with C-peptide to rats injected with the inflammatory agents thrombin or N^{ω}-nitro-L-arginine methyl ester (L-NAME), which cause acute endothelial dysfunction, resulted in reduced expression of intercellular cell adhesion molecule (ICAM)-1 and P-selectin on the mesenteric microvascular endothelium [28]. As a consequence, the number of rolling, adhering and transmigrated leucocytes also decreased upon C-peptide administration to the animals. In another model of vascular injury, systemic administration of C-peptide decreased polymorphonuclear leucocyte infiltration in isolated rat hearts following ischaemia–reperfusion injury and restored cardiac contractile function and postreperfusion coronary heart flow [27]. Our group has reported on the anti-inflammatory activity of C-peptide in high-glucose endothelial dysfunction, when C-peptide decreased vascular cell adhesion molecule 1 (*VCAM1*) mRNA expression and protein levels, and reduced secretion of IL-8 and monocyte chemoattractant factor (MCP)-1 by HAEC to the basal levels measured under normal glucose concentrations [29].

In this current study, we demonstrated that C-peptide reduced glucose-induced apoptosis of HAEC. Activation of caspase-3 is a central component of the proteolytic cascade in glucose-induced apoptosis of human endothelial cells [5]. In our model, we found that overnight exposure to high glucose increased levels and activity of activated caspase-3, which was reduced by addition of C-peptide to HAEC in vitro. Moreover, in agreement with Bugliani et al., who studied human pancreatic beta cells, levels of the anti-apoptotic molecule BCL-2, but not of the pro-apoptotic molecule BAX, were upregulated by C-peptide compared with high glucose alone in HAEC [40]. BAX belongs to the BCL-2 protein family of apoptosis-regulator gene products that may function as apoptotic activators (BAX, BCL2-antagonist/killer 1 [BAK], BCL2-associated agonist of cell death [BAD], and others) or facilitating cell survival (BCL-2, BCL2-like 1 [BCL-XL], BCL2-like 2 [BCL-W], and others) [41]. Although the protective effect of C-peptide in endothelial cell apoptosis is reported here for the first time, the anti-apoptotic effect of C-peptide has been already described in different cellular models. Using human neuroblastoma SH-SY5Y cells, Li et al. found that C-peptide reduced high-glucose-induced apoptosis by promoting the expression of *BCL-2* (also known as *BCL2*) [42]. In addition, in the BB/Wor rat model of spontaneous type 1 diabetes, C-peptide decreased hippocampal cell apoptosis, which was accompanied by lowered caspase-3 activation [43]. Finally, in this paper we show that C-peptide reduced TNF-α-induced HAEC apoptosis, decreased expression of caspase-3 and upregulated *BCL-2*. A similar result was reported by Al-Rasheed et al. in opossum kidney proximal tubular cells [34]. Taken together, these findings support the view that C-peptide prevents cellular apoptosis mediated by different inflammatory stimuli.

Although it has been shown that C-peptide acts via $G\alpha_i$ possibly via a G-protein-coupled receptor to protect against TNF-α-induced apoptosis in kidney proximal tubular cells [34], the intracellular mechanisms of C-peptide-mediated anti-apoptotic effects in endothelial cells are not well understood. In high-glucose-exposed endothelial cells, cellular apoptosis involves oxidative-stress-triggered activation of the NF-κB pathway [7, 10] which, in turn, suppresses BCL-2 levels and activates caspase-3 activity [10, 44]. We have previously observed that C-peptide interferes with glucose-induced nuclear translocation of the NF-κB p65/p50 subunits in HAEC, and reduces endothelial dysfunction [29]. An effect of C-peptide on NF-κB and consequent decreased inflammatory cytokine production has also been reported in the brain of diabetic BB/Wor rats and found to be associated with reduced neuronal apoptosis [31, 45, 46]. Here, we add significant pieces of information, by showing that C-peptide decreases intracellular ROS generation, a crucial upstream signalling event in the NF-κB pathway. In our model, ROS generation in HAEC was measured after overnight incubation with high glucose. C-peptide treatment quenched high-glucose-induced ROS production to bring levels closer to those detected in normal glucose at all time points, reaching

statistical significance at 3 h, thus suggesting that C-peptide exerts its beneficial effects on glucose-exposed endothelial cells over time. Our results are in apparent contrast with those from Stevens et al. [47], who reported no changes in antioxidant enzymatic activity in sciatic nerve homogenates from diabetic BB/Wor rats who were administered C-peptide for 2 months as compared with animals who did not receive C-peptide, although amelioration of endoneural nerve blood flow was found.

A possible explanation for these contrasting results could lie in the different methods used to detect oxidative stress and to the different experimental conditions employed in the two studies. While Stevens et al. determined levels of antioxidant enzymes in homogenates of rat sciatic nerves, we directly measured intracellular ROS production in live cultured HAEC after short exposure to high glucose. Thus, C-peptide might have different effects in different tissues under different experimental conditions. For example, one could speculate that timing of cellular exposure to C-peptide might be important as the most meaningful beneficial effects of C-peptide on oxidative stress are rapid, thus suggesting that C-peptide acts at the very early stages of glucose-induced vascular dysfunction. Furthermore, it might be that nerve cells and endothelial cells have different basal activities and mRNA levels of antioxidant enzymes so that one cell is more susceptible to oxidative stress than another. Antioxidant enzymatic activity of C-peptide-treated cells was not investigated in our study. In addition, the exact antioxidant enzymes that are induced by high glucose in HAEC and whether C-peptide is able to affect their mRNA levels or activities are not known.

We showed that C-peptide inhibits glucose-induced NAD(P)H oxidase activation, which is the major source of ROS in endothelial cells. This multi-component enzyme includes a membrane-bound cytochrome b_{558}, comprised of $p22^{phox}$ and $gp91^{phox}$ subunits, and the cytosolic adapter proteins $p47^{phox}$ and $p67^{phox}$, which are recruited to the cytochrome during stimulation to form a catalytically active oxidase [16, 17]. Recruitment of $p47^{phox}$ and $p67^{phox}$ to the plasma membrane requires the presence of RAC-1, a member of the rho family of small GTP-binding proteins that complex with the cytosolic proteins to regulate NAD(P)H oxidase activity. In this study, we report that glucose-induced RAC-1 protein levels at the plasma membrane of HAEC were reduced by 30 min treatment with C-peptide in vitro. Moreover, glucose-induced RAC GTPase activity was also reduced by C-peptide in HAEC. All together, these findings demonstrate that C-peptide decreases ROS generation by affecting RAC-1-dependent NAD(P)H oxidase activation in glucose-exposed HAEC. Thus, based on these findings we suggest that C-peptide in healthy individuals may represent an endogenous molecule with antioxidant properties that, once secreted in the bloodstream, protects the vascular endothelium from the damaging effects of hyperglycaemia-induced oxidative stress. An effect of C-peptide on preserving endothelial function by affecting indices of oxidative stress in individuals with diabetes was reported a few years ago by Manzella et al. [48].

How exactly C-peptide interferes with RAC-1-mediated NAD(P)H generation of ROS is not known. Based on our data, we support the hypothesis that C-peptide may interfere with translocation of RAC-1 from the cytoplasm to the membrane. In fact, membrane levels of RAC-1 and its GTPase activity were significantly reduced in C-peptide-treated endothelial cells. In our model, no effect by C-peptide on *RAC-1* mRNA gene expression was detected after 30 min exposure. Thus, we conclude that C-peptide may have an effect on post-translational modifications (i.e. isoprenylation) of RAC-1 that are required for translocation to the plasma membrane upon activation [49]. In addition, C-peptide may also affect translocation of the other NAD(P)H cytoplasmic subunits $p67^{phox}$ and/or $p47^{phox}$ which, when bound to RAC-1, can migrate from the cytoplasm to plasma membrane where activation of the cytochrome occurs. Further studies are necessary to investigate these theories.

We conclude that C-peptide prevents apoptosis in high-glucose-exposed HAEC by reducing oxidative stress. We have identified the RAC-1 pathway as a potential intracellular target of C-peptide in reducing ROS generation and apoptosis.

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Contribution statement V.C. designed and performed the majority of experiments, analysed and interpreted the data and critically revised the article. X.G. designed and performed the TUNEL assay, analysed the images, and critically revised the article. A.S. and R.L. performed the flow cytometry and analysed the experiments for ROS detection. They also critically revised the article. M.T. conceptualised the study, interpreted the data and critically revised the article. P.L. conceptualised the study, analysed and interpreted the data and wrote the article. All authors approved the final version of the paper.

Duality of interest statement The authors declare that there is no duality of interest associated with this manuscript.

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Review Article

C-peptide and long-term complications of diabetes

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C-peptide or connecting peptide is the 31-amino-acid segment that links the A and B chains of proinsulin and serves to promote the efficient folding, assembly and processing of the insulin molecule in the β-cell endoplasmic reticulum in the course of insulin biosynthesis (Fig. 1) (1). Equimolar amounts of insulin and C-peptide are subsequently stored in secretory granules of the β-cells and eventually released into the portal and systemic circulations. Unlike insulin, the C-peptide escapes hepatic retention and circulates at a concentration approximately 10-fold higher than that of insulin. The peptide is primarily catabolized by the kidneys and the biological half-life of the C-peptide is more than 30 min in adult humans, compared to 3–4 min for insulin (2).

Soon after its discovery in 1967, several investigators evaluated C-peptide for possible insulin-like effects but none were found. The apparent lack of physiological effects and consideration of the C-peptide structural variability and limited sequence conservation between species (3) led to the general view that the peptide was devoid of physiological effects other than its role in insulin biosynthesis (4). Instead, interest focused on the fact that it is co-secreted with insulin and that plasma concentrations of C-peptide effectively reflect the endogenous insulin secretion (5). As a marker of insulin secretion, C-peptide has been of great value in furthering our understanding of the pathophysiology of both type 1 diabetes (T1D) and type 2 diabetes (T2D). Even though C-peptide left the scientific limelight in the mid 1980s, interest in the possibility that the peptide may exert physiological effects remained. This notion was supported by the clinical observation of long standing that, compared to T1D patients in whom β-cell function ceases totally, those patients who retain a low but detectable level

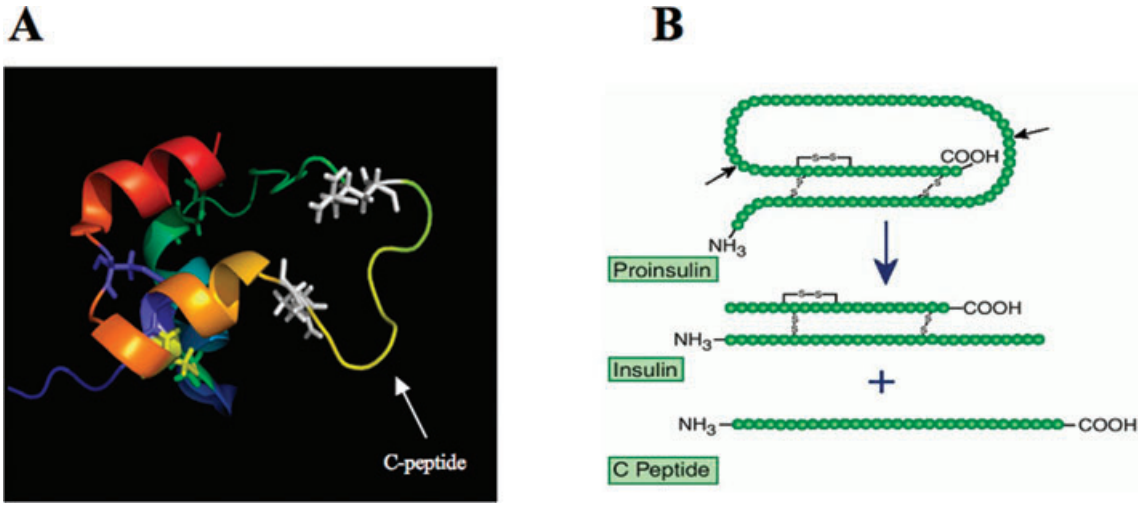

Fig. 1. Proinsulin C-peptide molecule. C-peptide is the peptide segment connecting insulin A and B chains, and a product of proinsulin cleavage in the secretory granules, generated in the pancreatic β-cells as part of normal insulin production. In **A**, a tridimensional image of the proinsulin molecule, from which C-peptide is cleaved by endoproteases at the level of the two Arginine residues linking the B-chain and C-peptide and at the level of the lysine–arginine residues linking the C-peptide and the A-chain (in white) is shown. C-peptide is then secreted into the bloodstream in equimolar amount with insulin in response to elevated blood glucose levels. In **B**, a schematic view of the proinsulin molecule is shown. The black arrows indicate sites of cleavage by proteases at the level of arginine residues. C-peptide circulates at low nanomolar concentrations in healthy individuals, but is absent in most patients with type 1 diabetes (T1D).

of C-peptide are less prone to develop microvascular complications of the eyes, kidneys, and peripheral nerves (6, 7). This view has recently received support from a study involving a large cohort of T1D patients treated uniformly at one medical center. The results show that a remaining C-peptide level above 0.06 nM confers a statistically significant protective effect against the development of microvascular complications independently of glycemic control, duration of diabetes, age, and sex (8). Moreover, pancreas or islet transplantation with restoration of endogenous insulin and C-peptide secretion are known to be accompanied by improvement of diabetes-induced abnormalities of nerve function, endothelial function, and both structural and functional changes of the kidneys (9, 10).

During the last 10–15 yr the above indirect evidence for physiological effects of C-peptide has been supported by a series of studies providing robust and direct evidence that C-peptide is in fact a biologically active peptide in its own right. In this review, we aim to present molecular studies showing binding of C-peptide to cellular membranes, activation of specific signaling pathways, and end effects of critical importance for several cell functions. In addition, clinical studies involving administration of C-peptide in replacement doses to T1D patients will be presented, which highlight beneficial effects on nerve and kidney function. Finally, new aspects of C-peptide physiology and therapeutic possibilities will be discussed, in particular, with regard to the anti-inflammatory characteristics of the peptide.

T1D as an inflammatory disease

Although T cells are recognized to play a central role in the autoimmune destruction of the insulin-producing β-cells (11), recent studies indicate that components of the innate immune system, including natural killer cells, monocytes, and inflammatory mediators have a much broader role in the pathogenesis of T1D and associated vascular complications than the previously recognized components (12–15). The primary role of monocytes in the early stages of T1D pathogenesis has been demonstrated by showing that these cells are the first to accumulate in the pancreatic islets of prediabetic BioBreeding (BB) rats (16). Subsequent T and B lymphocyte infiltration is dependent upon prior monocyte invasion of the islets (16), suggesting that monocytes and secreted inflammatory mediators might contribute to the early induction and amplification of the autoimmune assault against the pancreatic β-cells (17). A more generalized inflammatory response, with activation of monocytes and presence of oxidative stress has been found in the peripheral circulation of T1D patients. This inflammation is characterized by the elevation of plasma levels of several inflammatory biomarkers, such as interleukin (IL)-1β, IL-6, IL-8, tumor necrosis factor (TNF)-α, and C-reactive protein. Such inflammatory reactions have been detected both in recently diagnosed T1D children as well as in adult T1D patients well after the onset of diabetes (18–22). These findings demonstrate that a generalized inflammatory response is present already

in the very early stages of diabetes (23, 24). Many of the reported inflammatory changes are detected at the level of monocytes, which show upregulation of the adhesion molecule CD11b (Mac-1) (24) and have aberrant constitutive and lipopolisaccharide (LPS)-stimulated expression of cyclooxygenase (COX)-2, a defect which may predispose to a chronic inflammatory response in T1D (15). The vascular endothelium represents a likely target of this inflammatory response by inducing endothelial cell activation, alteration of endothelial function, and monocyte adherence eventually leading to overt vascular damage in the later stages of T1D. Indeed, inflammation is now considered a major component in the development of T1D-associated vascular dysfunction (20, 25, 26).

C-peptide and endothelial function

Under conditions of hyperglycemia or other inflammatory insults, endothelial cells become activated by changing the expression of adhesion molecules on the cellular surface, increasing secretion of pro-inflammatory cytokines, and allowing adhesion and migration of circulating monocytes and other leukocytes through the endothelial layer, a first step toward atherosclerosis plaque formation (Fig. 2) (27–29). C-peptide is now emerging as a molecule displaying potential beneficial effects on the dysfunctional endothelium, as shown in several *in vivo* and *in vitro* models of inflammation-mediated vascular injury. Although the mechanism(s) by which the C-peptide exerts its cytoprotective effects on the endothelium are not entirely understood, it has been reported that C-peptide can influence the activation of different signaling pathways that ultimately modulate or shut down inflammatory responses. In the sections below, we present recent findings in this area.

Effect of C-peptide on nitric oxide and blood flow

In recent years one of the most studied physiological effects of C-peptide is its action on the synthesis of biologically active substances secreted by endothelial cells in both physiological and pathological conditions. Nitric oxide (NO) represents one of these molecules, whose primary functions are to modulate the vascular tone and reactivity (30) and to downregulate inflammatory responses (31). The level of endothelial NO is tightly regulated to ensure maintenance

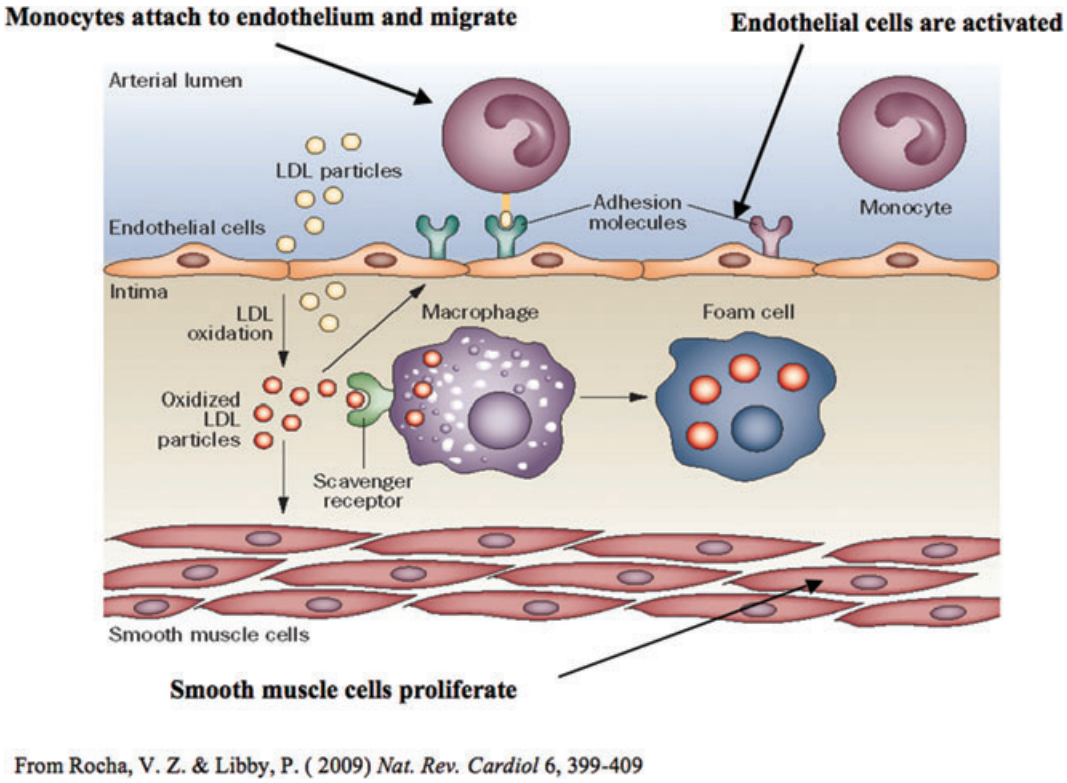

Fig. 2. Origin of vascular disease in diabetes. Exposure of endothelial cells to high glucose causes inflammatory changes culminating in the upregulation of cell adhesion molecules (i.e., vascular cell adhesion molecule (VCAM)-1) and secretion of pro-inflammatory cytokines i.e., interleukin (IL)-8 and monocyte chemoattractant protein (MCP)-1. As a consequence, monocytes and other circulating leukocytes adhere to the activated endothelial cells by interacting with the upregulated adhesion molecules. Subsequently, leukocytes migrate to the subendothelial space, phagocyte oxidizes LDL, and become foam cells. Smooth muscle cells proliferate and migrate from the media to the intima of the vessel wall where the new atherosclerotique plaque is developing.

of the adequate vascular tone and endothelial function.

Endothelial cells produce NO in response to elevation of intracellular Ca^{2+} concentrations, which in turn stimulate endothelial NO synthase (eNOS) (32). Under the hyperglycemic conditions typical of diabetes, Ca^{2+} entry in endothelial cells is impaired as a consequence of increased inflammation and oxidative stress (33). This impairment in Ca^{2+} homeostasis affects synthesis of NO (34) leading to attenuation in endothelium-derived vasodilation. *In vitro* exposure of endothelial cells to C-peptide induces release of NO (35–37), an effect responsible for the increased blood flow that C-peptide consistently causes in a number of tissues *in vivo*. In T1D patients, forearm blood flow, measured by venous occlusion plethysmography, showed a concentration-dependent increase in response to C-peptide infusion in the range 0–1 nM (38); no additional circulatory effect of C-peptide occurred when the concentration was raised above 1 nM, in keeping with the demonstration that the binding curve for C-peptide reaches saturation at approximately 1 nM (see below). The stimulatory effect of C-peptide on blood flow is abolished when an eNOS blocker is co-infused with the peptide (39), confirming that the effect is mediated via augmented NO release. The effects of C-peptide on the large artery endothelial function have been studied in T1D during reactive hyperemia, using ultrasound measurement of brachial artery blood flow and diameter (40). Shear stress-induced arterial dilatation, as evaluated by reactive hyperemia, was reduced in the patients. C-peptide administration resulted in a 35% increase in the basal blood flow but did not alter the reactive hyperemia, suggesting that C-peptide exerts its effects primarily on the distal resistance vessels. Forearm vascular dynamics and C-peptide effects have also been studied during exercise. Rhythmic forearm exercise in T1D patients during C-peptide infusion to physiological concentrations resulted in increased blood flow and capillary diffusion capacity, while vascular resistance decreased to levels similar to those for healthy subjects (41). These results and those from a study involving the rat hindquarter model (42) indicate that the effects of C-peptide on skeletal muscle involve facilitation of capillary recruitment.

The effects of C-peptide on blood flow have been demonstrated not only for forearm tissues, mostly skeletal muscle, but also for skin (43), kidney (44), peripheral nerve (45), and myocardium (46, 47). These findings are in keeping with the hypothesis of impaired endothelial function in T1D (48) that is partly corrected by augmented NO availability secondary to C-peptide replacement. The studies on effects of C-peptide on myocardial blood flow involved patients with T1D without signs of heart disease. The left ventricular myocardial blood flow was reduced in the basal state compared to healthy controls and C-peptide elicited marked increases in basal blood flow (46) and during adenosine-induced myocardial hyperemia (47). Both studies also demonstrated C-peptide mediated improvements in left ventricular performance, as indicated by augmented rates of both contraction and relaxation as well as by increased left ventricular ejection fraction and stroke volume, effects that may be related not only to increased myocardial blood flow but also to myocardial Ca^{2+} influx and Na^+,K^+–ATPase stimulation.

Besides being related to the NO-mediated direct effects on the resistance vessels, the circulatory effects of C-peptide also involve rheological factors. Erythrocyte deformability is known to be impaired in diabetes (49, 50), with potentially negative effects on the microcirculation. It now emerges that C-peptide is able to ameliorate the altered erythrocyte deformability in T1D. This effect is blockable by ouabain, indicating that it is mediated by a restoration of erythrocyte Na^+,K^+–ATPase activity (51).

Effect of C-peptide on inflammation and endothelial dysfunction

Several findings support the idea that C-peptide affects leukocyte–endothelium interactions by reducing upregulation of cell adhesion molecules typically observed under inflammatory conditions. The first evidence of this effect is from Scalia et al. (36), who demonstrated that pretreatment with C-peptide to rats injected with the inflammatory agents thrombin or N^G-nitro-L-L-arginine methyl ester (L-NAME), causing acute endothelial dysfunction, resulted in reduced expression of ICAM-1 and P-selectin on the mesenteric microvascular endothelium. As a consequence, the number of rolling, adhering, and transmigrated leukocytes also decreased upon C-peptide administration to the animals. In another model of vascular injury, C-peptide decreased polymorphonuclear leukocyte (PMN) infiltration in isolated rat hearts following ischemia-reperfusion injury (52). PMN infiltration induces endothelial and myocardial injury by releasing cytotoxic substances such as oxygen-derived free radicals, inflammatory cytokines, and proteolytic enzymes. By reducing PMN infiltration to the myocardium, C-peptide restored cardiac contractile function and postreperfusion coronary heart flow (52). These findings have been recently recapitulated *in vitro* in a model of high glucose-endothelial dysfunction in which adhesion of the monocytic cell line U-937 to high glucose-stimulated human aortic endothelial cells (HAEC) *in vitro* decreased by 50% after addition of physiological concentrations of C-peptide, an effect not detected

when C-peptide was heat-inactivated (53). C-peptide was shown to reduce expression of both VCAM-1 mRNA and protein expression in high glucose-treated HAEC. In the same model, C-peptide was also demonstrated to reduce high glucose-induced secretion of IL-8 and MCP-1 by HAEC to the basal levels measured under normal glucose concentrations (53). These two chemokines are essential to promote leukocyte adhesion to endothelial cells. Conversely, when C-peptide was added to the medium containing normal glucose levels, it failed to significantly reduce VCAM-1 expression and IL-8 or MCP-1 secretion from HAEC suggesting that the most meaningful biological effects of C-peptide on the endothelium are observable under conditions of vascular insult or damage.

Potential intracellular pathways mediating the anti-inflammatory effects of C-peptide

The nuclear factor -κB pathway

A likely candidate for C-peptide-mediated anti-inflammatory effects in the vasculature is represented by the signal transduction pathway requiring translocation of the transcription factor nuclear factor (NF)-κB, a major player in mediating inflammatory responses in a variety of cells (54). In the unstimulated state, NF-κB exists as a heterodimer composed of p50 and p65 subunits bound to IκB in the cytoplasm (Fig. 3). Upon activation, for example after cellular exposure to high glucose, IκB is phosphorylated and degraded, thus causing release of the p50/p65 components of NF-κB. The active p50/p65 heterodimer translocates to the nucleus and initiates the transcription of a gamut of genes involved in the inflammatory response, such as pro-inflammatory cytokines, cell-surface adhesion molecules, and chemokines, including IL-8 and monocyte chemoattractant protein (MCP)-1 (Fig. 3) (54). In a recently published paper, it was demonstrated that physiological concentrations of C-peptide reduce high glucose-induced activation of NF-κB in cultured HAEC, by decreasing translocation of the NF-κB canonical components p65 and p50 into the nucleus (53). By reducing NF-κB nuclear translocation, C-peptide might reduce adhesion molecule expression as well as secretion of inflammatory cytokines, such as IL-8 and MCP-1 in cultured HAEC (53). It is not known which NF-κB-dependent upstream signaling events are affected by C-peptide in endothelial cells; examples are reactive oxygen species (ROS) generation and IκB kinase, an enzyme that elicits phosphorylation of the cytosolic NF-κB inhibitor IκBα. This latter upstream event regulates NF-κB translocation from the cytoplasm to the nucleus. In vascular smooth muscle cells (VSMC), C-peptide reduces high glucose-induced proliferation; a key event in atherogenesis, by reducing phosphorylation of IκBα, a pathway likely to be

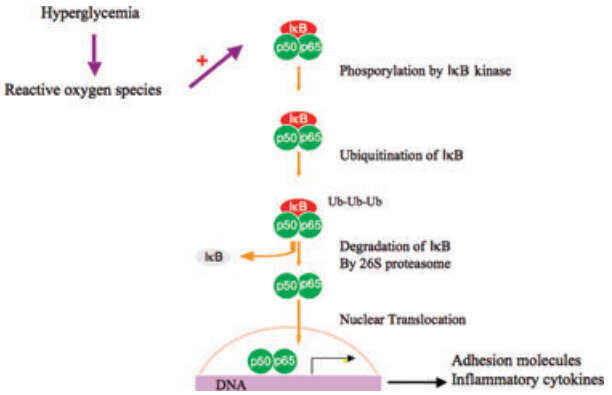

Fig. 3. The nuclear factor (NF)-κB is a potential target for the anti-inflammatory activity displayed by C-peptide. NF-κB exists as a heterodimer composed of p50 and p65 subunits bound to IκB in the unstimulated state. Upon cellular activation, i.e., after exposure of endothelial cells to high glucose, increased production of reactive oxygen species (ROS) mediates phosphorylation and degradation of IκB, thus causing the release of p50/p65 components of NF-κB. The active p50/p65 heterodimer translocates to the nucleus and initiates the transcription of genes involved in the regulation of leukocyte responses, such as pro-inflammatory cytokines and cell-surface adhesion molecules expression. C-peptide decreases nuclear translocation of p65/p50 subunits in high glucose-activated endothelial cells (53). C-peptide might act at different levels along the NF-κB pathway, such as regulating ROS production, or modulating IκB phosphorylation, or even physically interacting with the p65/p50 subunits in the nucleus preventing their DNA binding.

targeted in endothelial cells (55). Another possibility is that C-peptide directly interacts with NF-κB p65/p50 subunits at the nuclear level, preventing DNA binding.

Other studies also demonstrate the importance of C-peptide in modulating the NF-κB activation and inflammation in the central nervous system. T1D patients may suffer impairments in learning, memory, problem solving, and mental and motor speed with primary diabetic encephalopathy recognized as a late complication of T1D (56). In the type 1 BB/Worcester (BB/Wor) rats (rat model of human T1D), cognitive impairment is associated with apoptosis-induced neuronal loss in the hippocampus, an event associated with NF-κB and receptor for advanced glycation end products (RAGE) activation (56). C-peptide replacement therapy to these diabetic rats reduced NF-κB and RAGE expression in the hippocampi leading to a decreased production of inflammatory cytokines, such as TNF-α, IL-1β, IL-2, and IL-6 (56) and prevention of the cognitive dysfunction and hippocampal neuronal loss (57).

The peroxisome proliferator-activated receptor γ pathway

Another signaling pathway that is affected by C-peptide is the one mediating activation of peroxisome proliferator-activated receptor γ (PPAR-γ) (58), a member of the nuclear receptor superfamily

of ligand-activated transcription factors. In addition to its function in adipogenesis and increasing insulin sensitivity, PPAR-γ regulates the expression of several genes involved in inflammation and vascular disorders, such as atherosclerosis (59), by either controlling the gene transcriptional machinery or interacting with other transcription factors such as activator protein (AP)-1, signal transducers and activators of transcription (STAT), and NF-κB (60). By neutralizing NF-κB activation, PPAR-γ modulates a constellation of inflammatory events crucial for the initiation of vascular diseases (61). Although the effect of C-peptide on the activation of PPAR-γ has not been investigated in the vasculature, there is evidence that C-peptide decreases systemic inflammation in an animal model of sepsis by increasing DNA binding of PPAR-γ in the lung of endotoxin-treated mice, an event associated with inhibition of the phosphorylation of extracellular signal-regulated kinase (ERK)-1/2 (62).

C-peptide and nerve function in T1D

Diabetic polyneuropathy, the most common microvascular complication of diabetes mellitus, occurs in both T1D and T2D (63). In T1D, the neuropathy tends to progress more rapidly and results in a more severe disorder than in T2D (64). The underlying pathogenetic mechanisms are multiple and thought to involve genetic predisposition as well as metabolic abnormalities consequent to hyperglycemia, such as oxidative stress, accelerated polyol pathway metabolism and generation of advanced glycosylation end products (65). The available data suggest that in the case of T1D other factors, notably C-peptide deficiency, may also play a role (66). In the diabetes control and complications trial (DCCT) study it became evident that intensive as compared to conventional insulin therapy markedly reduces the development of clinical neuropathy (67). Yet, in the intensively treated group, with near-normal blood glucose levels, the cumulative prevalence of overt neuropathy and/or grossly abnormal nerve conduction after 5 yr approached 40%, pointing at the possibility that factors other than hyperglycemia also contribute to the progressive deterioration of nerve function in this disorder. Further support for this view is provided by studies of nerve function and structure in animal models of diabetes and nerve biopsy samples from patients. The nerve abnormalities in type 1 animals, who lack C-peptide, include impairment of nerve Na^+,K^+–ATPase, and eNOS activities, resulting in intra-axonal sodium accumulation and reduced endoneurial blood flow (66). Gradually, structural changes appear, involving axonal atrophy and characteristic nodal and paranodal abnormalities that contribute to the progressive deterioration of nerve conduction velocity (NCV) (68, 69). In contrast, in T2D, with normal or elevated levels of C-peptide and where hyperglycemia is the primary pathogenetic factor, the functional and structural abnormalities of the peripheral nerves are less marked and show a different pattern, including milder axonal degeneration and no or only minimal nodal and paranodal abnormalities (70, 71). The discrepancies in diabetes-induced structural changes between T1D and T2D are also demonstrated in nerve biopsy samples from patients, where the structural abnormalities in T2D patients follow the normal pattern of ageing whereas patients with T1D present with significant nodal and paranodal structural changes (68). Thus, it is conceivable that the lack of C-peptide in T1D contributes to the development of the more severe nerve dysfunction and structural abnormalities in this disorder.

Clinical studies

C-peptide replacement was given for 3 months in a double-blind, placebo-controlled study including 46 patients with approximately 10-yr diabetes duration and reduced sensory and motor nerve conduction velocities (NCV) but no overt symptoms or signs of neuropathy (72). Sensory (sural) but not motor (peroneal) NCV increased gradually during the study; the increase after 3 months was 2.7 m/s, corresponding to an 80% correction of the initial deficit (Fig. 4). Vibration perception thresholds showed only a small increase above normal at baseline but decreased significantly during treatment, consistent with an improved sural nerve function.

The above observations have been extended in a subsequent double-blind clinical trial involving 161 T1D patients with manifest diabetic peripheral neuropathy (73). The patients received either a replacement dose of C-peptide, a dose three times higher or placebo. Sensory NCV improved similarly

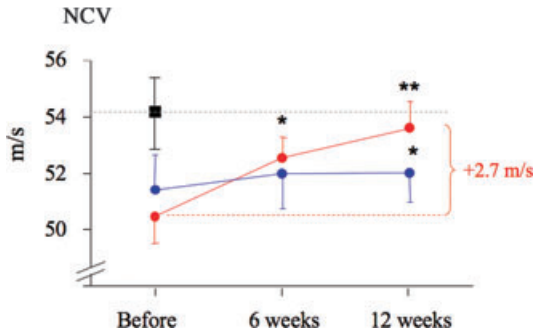

Fig. 4. C-peptide and sensory nerve conduction velocity (NCV) in patients with type 1 diabetes (T1D) and early stage neuropathy. Change in sural NCV after 6 and 12 wk of C-peptide treatment (red symbols) or placebo administration (blue symbols). ** indicates $p < 0.01$ for the increase above baseline NCV in the C-peptide-treated group. The difference between the C-peptide and placebo groups at 12 wk was statistically significant ($p < 0.05$). Scr = Scrambled C-peptide. Adapted from Ref 38.

within the two C-peptide-treated groups and the number of positive responders was significantly greater among the patients receiving C-peptide ($p < 0.03$). The improvement in comparison to placebo was approximately 1 m/s in the least severely diseased half of the patients ($p < 0.02$). The neurological impairment score and vibration perception also improved within the C-peptide-treated group. Glycemic control improved slightly but similarly in the three study groups. In keeping with these findings, an improvement in temperature perception thresholds has been reported after 3 months of C-peptide replacement therapy (74). Metabolic control and blood pressure were unchanged in all of the above studies, indicating that these factors were not responsible for the improvement. The observed improvement in sensory NCV and vibration and temperature perception may be seen as favourable considering the relatively short treatment periods and previous experience of aldose reductase inhibitors (75).

Deficient autonomic nerve function may be evaluated in patients as reduced heart rate variability (HRV) during deep breathing, a measurement that, with a high degree of reproducibility, primarily reflects vagal function. In T1D patients, autonomic dysfunction can be ameliorated by C-peptide in replacement doses; short-term infusion of C-peptide is reported to significantly increase HRV, while no change was seen after saline infusion (76). The heart rate brake index after a tilting maneuvre was also improved after C-peptide for 3 h. A similar, though less marked improvement was seen after 3 months of C-peptide administration in T1D patients (74).

Animal studies

Positive effects of C-peptide on motor and sensory NCV have been demonstrated in two animal models of T1D. In BB/Wor rats, showing spontaneous development of type 1-like diabetes, C-peptide administration (homologous C-peptide in replacement dose by continuous subcutaneous infusion) prevents the development of NCV deficits when the peptide was given from 1 wk after the onset of diabetes (Fig. 5) (77, 78). In addition, C-peptide elicited an increase in NCV and partially corrected the NCV deficit when treatment was commenced at a time when diabetes-induced abnormalities had become established (after 5 months of diabetes) (Fig. 5) (77). The C-peptide concentrations reached in these studies were in the low physiological concentration range (0.5–0.7 nM). Similarly, in streptozotocin-diabetic rats receiving rat C-peptide in replacement doses from 6 to 8 wk after induction of diabetes, the peptide gave rise to 80 and 60% corrections of the sensory (saphenous) and motor (sciatic) NCV, respectively (45). Scrambled

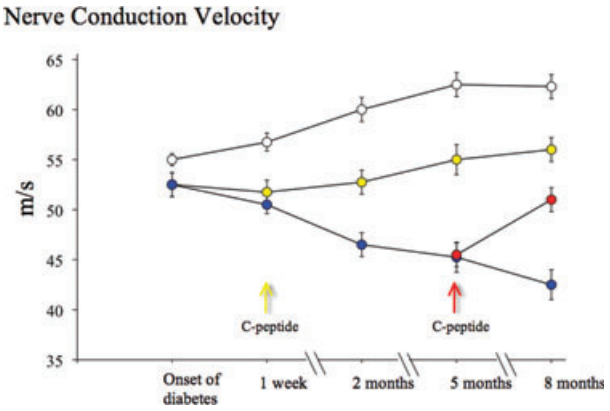

Fig. 5. C-peptide and nerve conduction velocity (NCV) in diabetic Biobreeding/Worcester (BB/Wor) rats. NCV in diabetic (blue symbols) and healthy (open symbols) BB/Wor rats given rat C-peptide in replacement dose by subcutaneous pump infusion starting 1 wk after onset of diabetes (yellow symbols) or after 5 months of diabetes (red circles). The NCV declined progressively in the untreated diabetic animals ($p < 0.01$), but increased in the C-peptide infused animals ($p < 0.05$–0.01). Data from Ref 77.

C-peptide, a control peptide with the same 31 residues assembled in random order, was without effect.

Several factors may contribute to the observed effect of C-peptide on NCV. Direct measurements of nerve blood flow, using the hydrogen clearance technique, have demonstrated that endoneurial blood flow is substantially reduced both in diabetic BB/Wor rats and in streptozotocin-diabetic rats (Fig. 6) (45, 79). Continuous-rate subcutaneous infusion of rat C-peptide in replacement doses for 2 wk or 2 months resulted in 52 and 75% correction, respectively, of the endoneurial perfusion deficit (Fig. 6). C-peptide effects on both endoneurial blood flow and NCV were abrogated by an eNOS blocker and the scrambled C-peptide had no effect. Altogether, the findings indicate that C-peptide in physiological

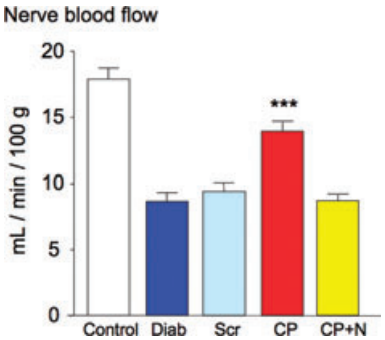

Fig. 6. C-peptide and nerve blood flow in diabetic rats. Endoneurial blood flow in streptozotocin-diabetic rats was measured using a hydrogen elimination technique. Findings are shown for healthy control rats (open bar), for diabetic untreated rats (blue bar), diabetic rats treated with C-peptide (red bar), and diabetic rats given both C-peptide and an endothelial nitric oxide synthase (eNOS) blocker NG-nitro-L-arginine (L-NNA) (yellow bar). Adapted from Ref 45.

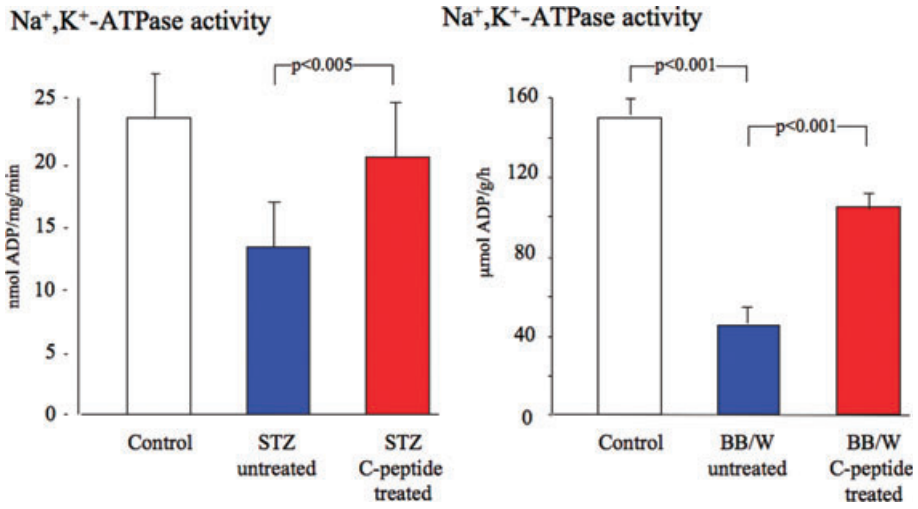

Fig. 7. C-peptide and nerve Na^+,K^+–ATPase activity in diabetic rats. Sciatic nerve Na^+,K^+–ATPase activity in healthy control rats (open bars), untreated diabetic rats (blue bars), and animals that received C-peptide for 5 wk after induction of diabetes (red bars) (left panel, streptozotocin-diabetic rats) or 2 months after diabetes onset (right panel, Biobreeding/Worcester (BB/Wor) rats). Data from Refs 77 and 80.

concentrations improves nerve function in T1D via a NO-sensitive vascular mechanism mediating vasodilation of the nerve blood vessels. In contrast to the above findings, a previous study has suggested that C-peptide reduces total nerve blood flow in streptozotocin-diabetic rats (80). However, the method for estimation of nerve blood flow was based on the microsphere entrapment technique, which has technical limitations when used in a small tissue such as the rat sciatic nerve (81).

Additional effects of C-peptide, demonstrable in *in vivo* animal studies, may contribute to its beneficial influence on nerve function. Decreased Na^+,K^+–ATPase activity in peripheral nerve tissue is a characteristic abnormality in T1D. It is associated with increased inactivation of Na^+-channels, intra-axonal sodium accumulation (82), and swelling of the paranodal region during the early phase of the disorder (83). C-peptide in physiological concentrations prevents or partially corrects the diabetes-induced reduction in nerve Na^+,K^+–ATPase activity both in streptozotocin-diabetic animals (80) and in BB/Wor rats (77), thereby contributing to improved electrolyte balance and partial correction of the paranodal swelling (Fig. 7) (77). At a later stage in the development, structural changes involving axonal atrophy and abnormalities of the nodal and paranodal apparatus occur (84). These include a progressive disruption of the paranodal myelin sheath, resulting in lateralization of the Na^+ ion channels of the large myelinated fibres, termed axoglial dysjunction, and eventually resulting in paranodal demyelination (84). The latter changes occur to a much lesser extent, or not at all, in T2D (70), and recent evidence suggests that they are the result of impaired C-peptide action rather than hyperglycemia (78). Accordingly, C-peptide treatment of diabetic BB/Wor rats resulted in marked improvements in nodal, paranodal, and axonal structural changes and in increased repair activity. Thus, 8 months of C-peptide treatment resulted in near total prevention of axoglial dysjunction and paranodal demyelination (85). Likewise, marked improvements in structural abnormalities were observed when C-peptide was given from 5 to 8 months after disease onset; axoglial dysjunction and paranodal demyelination improved significantly, axonal degeneration decreased and nerve fibre regeneration increased fourfold (77). A schematic representation of the different mechanisms, whereby C-peptide may exert beneficial effects on peripheral nerve dysfunction and structural abnormalities, is shown in Fig. 8.

Painful neuropathy is a debilitating consequence of diabetes, which is at least partly a consequence of

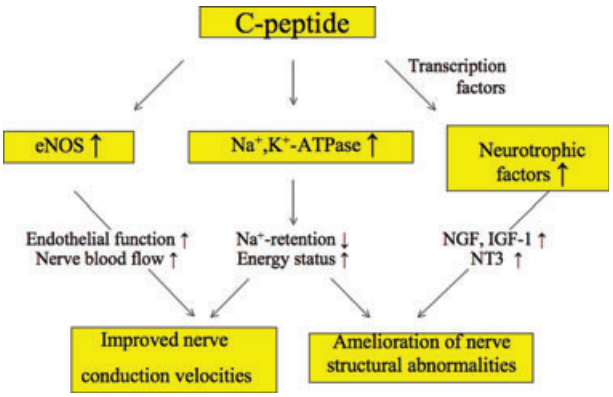

Fig. 8. C-peptide effects on nerve function and structure in diabetes. Schematic representation of the different mechanisms, whereby C-peptide may improve nerve function and ameliorate nerve structural abnormalities secondary to type 1 diabetes (T1D).

damage to the unmyelinated and the small myelinated nociceptive fibres (86) and to reduced neurotropic support by, e.g., nerve growth factor (NGF). Degeneration of these fibres initially leads to high-firing frequencies and spinal sensitization, which is experienced by the patient as hyperalgesia. Replacement of C-peptide from the onset of diabetes in rats completely prevents thermal hyperalgesia as well as degeneration and loss of unmyelinated fibres (87). These findings are accompanied by improved regulation of gene expression of different neurotropic factors, e.g., NGF and insulin-like growth factor-1 (IGF-1), and their receptors in the dorsal root ganglion of the rats after 8 months of treatment. Experimental evidence thus supports the notion that C-peptide may be useful in alleviating painful neuropathy in animals but clinical trials will be required to determine if this is true for the human painful neuropathy.

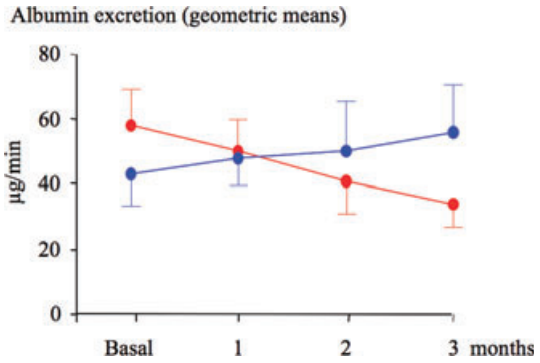

Fig. 9. C-peptide and albumin excretion in urine in type 1 diabetic (T1D) patients. Urinary albumin excretion (geometric means) in T1D patients with early stage nephropathy. The patients received C-peptide plus insulin (red symbols) or placebo plus insulin (blue symbols) for 3 months. Albumin excretion was significantly different between C-peptide and placebo-treated patients after 2 ($p < 0.05$) and 3 ($p < 0.01$) months, respectively. Data from Ref 74.

C-peptide and the kidneys in T1D

Early signs of diabetic nephropathy include glomerular hyperfiltration and loss of renal functional reserve, findings which are accompanied by renal hypertrophy and glomerular enlargement due to mesangial matrix expansion. Within a few years the structural changes develop further, glomerular expansion continues and, in addition, there is thickening of the basement membrane (88). An early sign is microalbuminuria (30–300 mg/24 h), frequently in combination with hypertension. Subsequently, the albumin excretion may accelerate and the condition develops into overt diabetic nephropathy with gradually decreasing glomerular filtration and macroalbuminuria (>300 mg/24 h). The prevalence of nephropathy is higher in T1D than in T2D patients and end-stage renal insufficiency is more common in T1D (89). In the former group, proteinuria is consistently accompanied by advanced glomerular structural changes, whereas there is no clear-cut link between urinary albumin excretion and glomerulopathy in T2D patients (90).

Studies in patients with T1D

The short-term effects of C-peptide administration on renal functions have been studied in young T1D patients without overt signs of renal disease (44). C-peptide, infused at rates sufficient to achieve physiological plasma concentrations, resulted in a decreased glomerular filtration rate and slightly increased renal plasma flow. These observations have been extended in a double-blind randomized study in T1D patients with incipient nephropathy receiving C-peptide for 4 wk (91). After 2 and 4 wk in the group receiving C-peptide, the glomerular filtration rate had decreased and at the end of the study there was a significant reduction in albumin excretion to approximately half the basal value (Fig. 9).

The above findings have been further explored in a study involving C-peptide administration for 3 months (74). A double-blind, placebo-controlled, randomized, crossover study design was used and patients with early stage nephropathy received C-peptide plus insulin and insulin plus placebo for 3 months. Pre-study urinary albumin excretion rates were on average 60 µg/min and all patients were normotensive. During the C-peptide-treatment period, urinary albumin excretion decreased progressively to values significantly below those observed during the pre-study period. The reduction was significant after 2 months, and at the end of the 3-month study period the decrease amounted to approximately 40%. When the patients received insulin only, albumin excretion did not change significantly. All patients remained normotensive throughout the study and glycemic control improved slightly but to the same extent during the two treatment periods. Taken together, the evidence supports the view that C-peptide in replacement doses has the capacity to reduce glomerular hyperfiltration and to decrease urinary albumin excretion in early stage T1D nephropathy. Needed now are long-term studies in patients with clinically manifest nephropathy to further define C-peptide's therapeutic potential in this disorder.

Animal studies

The influence of C-peptide on renal function has been evaluated in streptozotocin-diabetic rats (92). The animals showed glomerular hyperfiltration and urinary protein leakage prior to C-peptide administration. Renal functional reserve, evaluated during intravenous glycine infusion, was reduced in the diabetic compared

to normal rats. Short-term (90 min) C-peptide infusion was followed by an immediate and almost full correction of the glomerular hyperfiltration. The renal functional reserve was almost completely restored and urinary protein excretion decreased significantly (92). The specificity of the C-peptide effect was attested to by the finding that scrambled C-peptide had no effect.

The influence of more prolonged C-peptide administration on renal function and morphology has also been examined by Samnegard et al. (93). When C-peptide was given intravenously as replacement dosage for 14 days, the glomerular filtration rate and renal functional reserve were almost completely normalized and urinary albumin excretion decreased significantly in the C-peptide-treated rats and was similar to that in the controls. Examination of renal morphology revealed that C-peptide significantly diminished the diabetes-induced increase in glomerular volume; in the C-peptide-treated rats, the glomerular volume exceeded that of the controls by no more than 23% (ns) compared with 63% in the untreated diabetic group. When increasing doses of human C-peptide were administered to diabetic rats, C-peptide was found to dose-dependently lower the augmented glomerular filtration rate and albumin excretion. Renal function in healthy animals was unresponsive to C-peptide infusion (94). Further examination of the glomeruli revealed that it was primarily the diabetic-induced hypertrophy of the glomeruli and in particular the mesangial matrix that was inhibited by C-peptide (95).

Several recent studies have offered clues as to the physiological mechanism(s) involved in the beneficial effects of C-peptide on renal function and structure in diabetic nephropathy. Thus, C-peptide has been found to elicit a constriction of the afferent glomerular arteriole and a relaxation of the efferent arteriole in diabetes (96, 97). In addition, C-peptide administration results in an inhibition of tubular Na^+ reabsorption (96). Taken together, these effects will contribute to a reduction of the elevated glomerular filtration rate and a diminished urinary albumin excretion. The extent to which these effects are mediated by C-peptide's influence on either or both of renal eNOS (98) and glomerular and tubular Na^+,K^+–ATPase (99, 100) is not known. Early indications of the background to C-peptide's beneficial effects on diabetes-induced renal structural abnormalities have been observed in diabetic mice; C-peptide has been found to reduce the glomerular expression of the profibrotic cytokine transforming growth factor-beta (TGFβ) and type IV collagen (101). Moreover, C-peptide has been found to reverse the structural changes of tubular cells induced by TGFβ (102). Overall, there is much evidence in support of a renoprotective effect of C-peptide in the nephropathy of T1D.

How C-peptide works

Membrane binding and internalization of C-peptide

Although C-peptide exerts a variety of effects in different cell types, relatively little is known regarding exactly how C-peptide achieves its intracellular activities in target cells. It was initially thought that C-peptide exerted its effects via nonchiral mechanisms rather than by stereo-specific receptors or binding sites (80), although specific binding of C-peptide to cultured rat pancreatic β-cells was demonstrated in 1986 (103). Subsequently, stereo-specific binding of C-peptide to cellular membranes has been confirmed in several human cell types including human renal tubular cells, human fibroblast and saphenous vein endothelial cells (Fig. 10) (104, 105). Furthermore, C-peptide binding reaches full saturation at 0.9 nM; thus, in healthy subjects, receptor saturation is already achieved at physiologic levels (105). Although a putative C-peptide receptor has not been identified on human cell membranes, it has been suggested to be a G-protein-coupled receptor, as deduced from the inhibitory effects of pertussis toxin on C-peptide binding and intracellular signaling (105, 106).

More recently, C-peptide was shown to cross plasma membranes, localizing in the cytoplasm of HEK-293 cells and Swiss 3T3 fibroblasts (106), where it was detected up to 1 h after its uptake. Nuclear localization of C-peptide in HEK-293 cells and Swiss 3T3 fibroblasts has also been demonstrated by the same group (106). Specifically, C-peptide can be detected in the nucleoli where it promotes transcription of genes encoding for ribosomal RNA (107). Luppi et al. (108) also investigated the process of internalization of C-peptide and its subcellular localization in live HAEC

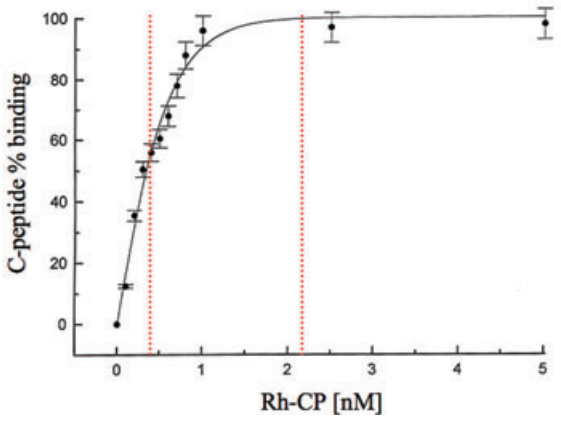

Fig. 10. Binding of rhodamine-labelled C-peptide to all membranes of the renal tubular cells. Fractional saturation of the membrane-bound ligand is presented as a function of the C-peptide concentration in the surrounding medium. The area between the red dotted lines represents the physiological concentration range. Data from Ref 105.

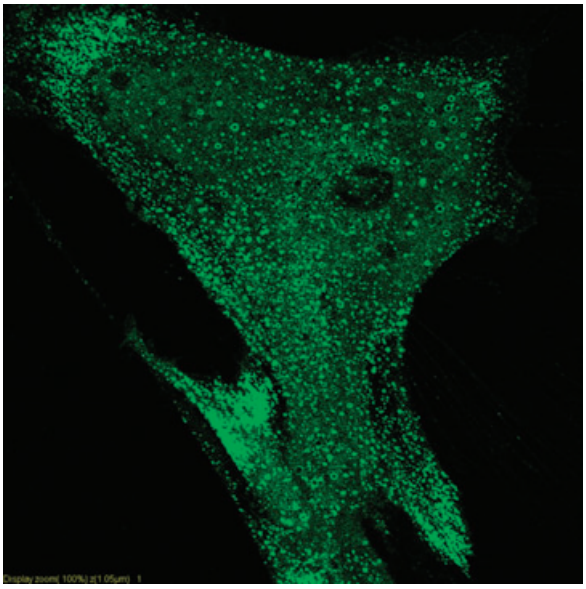

Fig. 11. C-peptide internalizes in human vascular endothelial cells as punctate structures. Internalization of C-peptide has been studied by using AlexaFluor488-labeled C-peptide probe in live-cell imaging using a laser scanning confocal microscopy (108). The green punctate-staining corresponds to the C-peptide probe localized at the periphery of the cell and in the cytoplasm.

and umbilical artery smooth muscle cells (UASMC) by using confocal laser scanner microscopy. They found that C-peptide internalizes to punctate structures localized at the level of the cellular membrane and in the cytoplasm (Fig. 11) (108). Internalization of C-peptide was minimal after 5 min, clearly detectable after 10 min, resulted in bright staining after 30 min, and was completed by 1 h (Fig. 11) (108). By using specific organelle reporter dyes, it was established that the punctate structures identifies with the specific endocytic organelles early endosomes. C-peptide eventually trafficked to lysosomes in live HAEC and UASMC (108). Identification of the subcellular compartments in which C-peptide localizes upon its entry into target cells is important as these compartments likely represent the sites of intracellular signaling activity of C-peptide. In one scenario, it can be envisaged that C-peptide after its binding to the specific receptor at the level of the plasma membrane gets activated, begins the signaling activity, and is quickly internalized into early endosomes where it continues to signal. Localization of C-peptide to early endosomes does not exclude trafficking of C-peptide to other subcellular sites upon its entry into target cells. As an alternative scenario, the signaling activity of the activated C-peptide–receptor complexes is localized at the plasma membrane. Internalization of the C-peptide–receptor complexes to early endosomes just represents a sorting station for internalized activated receptor–peptide complexes on their way to lysosomal degradation.

C-peptide and intracellular signaling

When exposed to C-peptide in physiological concentrations, renal tubular and endothelial cells show a prompt elevation of intracellular Ca^{2+} concentrations (Fig. 12) (35, 99, 109). C-peptide also elicits phosphorylation of phospholipase C (PLC) and several protein kinase C (PKC) isoforms, notably, α, δ, and ε, in different tubular cells and fibroblasts (100, 110, 111). PI3-kinase activation has been observed in fibroblasts, myoblasts, renal tubular cells, and lymphocytes (58, 112, 113). Activation of one or several components of the mitogen-activated protein kinase (MAP-kinase) system mostly via Rho A is consistently observed in all examined cell types following exposure to C-peptide at physiological concentrations (3, 110–112, 114, 115). A schematic overview of C-peptide signaling is shown in Fig. 13.

C-peptide has been found to mimic insulin effects in myoblasts and neuroblastoma cells by increasing autophosphorylation of the insulin receptor, stimulation of phosphoinositide 3 (PI3)-kinase but not Akt and phosphorylation of MAP-kinase (112, 116). Glucose utilization and glycogen synthesis are stimulated in myoblasts (112) and in human skeletal muscle strips (117). The mechanism by which these effects are elicited is not apparent; besides the binding of C-peptide to a specific G-protein-coupled receptor causing activation of PI3-Kγ, there could be crosstalk between insulin and C-peptide ligand-receptor complexes or interaction between C-peptide and receptors with catalytic activity, as suggested by the finding that C-peptide attenuates the activity of protein tyrosine phosphatase in myoblasts (112).

Endothelial nitric oxide synthase (eNOS)

There is *in vitro* evidence that C-peptide elicits release of NO in endothelial cells in a concentration and time-dependent manner (35). The effect, which has a rapid onset, is abolished in a Ca^{2+}-free medium and in the presence of an NO synthase inhibitor, suggesting that stimulation of eNOS via a Ca^{2+}-dependent signal is involved. In addition, increased expression of eNOS mRNA (36) and eNOS protein (37) has been observed after exposure of lung and aortic endothelial cells to C-peptide; eNOS expression is enhanced via a MAP-kinase-dependent transcriptional activation (37). The above *in vitro* observations are consistent with the finding that C-peptide administration to T1D patients and in animal models of T1D results in concentration-dependent increases in blood flow in skeletal muscle (38, 41), skin (43), peripheral nerve (45, 79), and myocardium (46, 47). The observations support the hypothesis that C-peptide administration in T1D partly corrects the diabetes-induced abnormality in eNOS activity and expression.

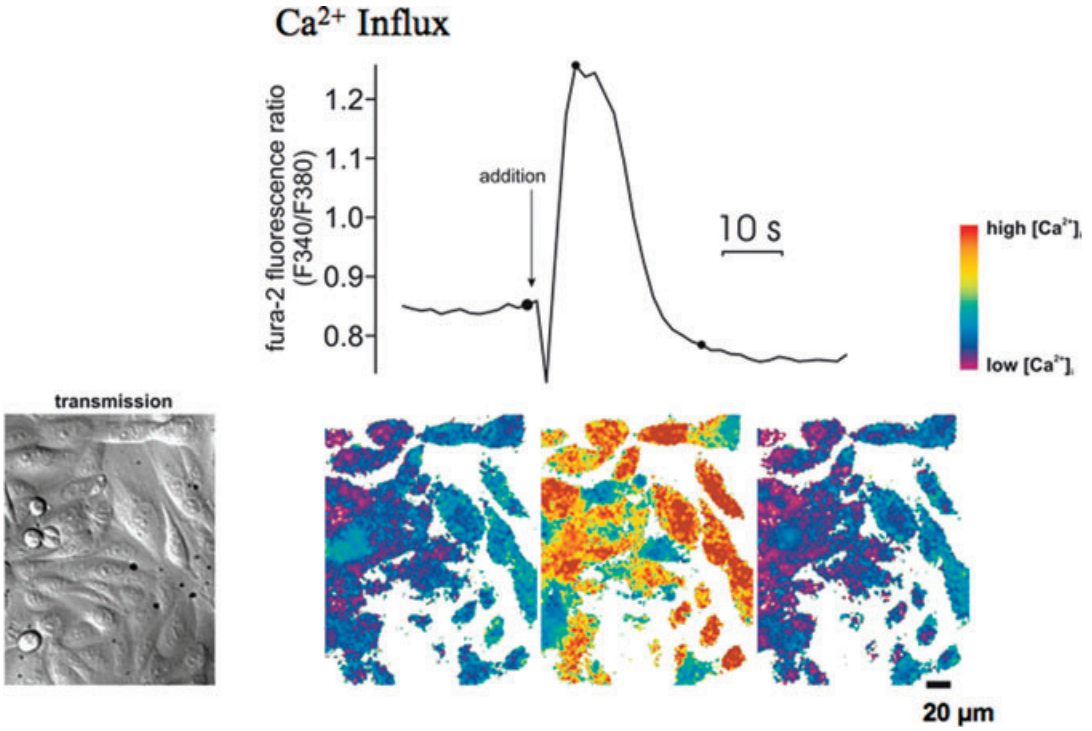

Fig. 12. Monitoring of $[Ca^{2+}]i$ in fura-2/AM-loaded human renal tubular cells stimulated with 5 nM human C-peptide. Top panel: the trace of the 340/380 fluorescence ratio. Bottom panel: images of the cells in transmission light (first panel) and in a color code (next three panels) representing $[Ca^{2+}]i$ at the time points shown by spot indications in the trace above. From Ref 109.

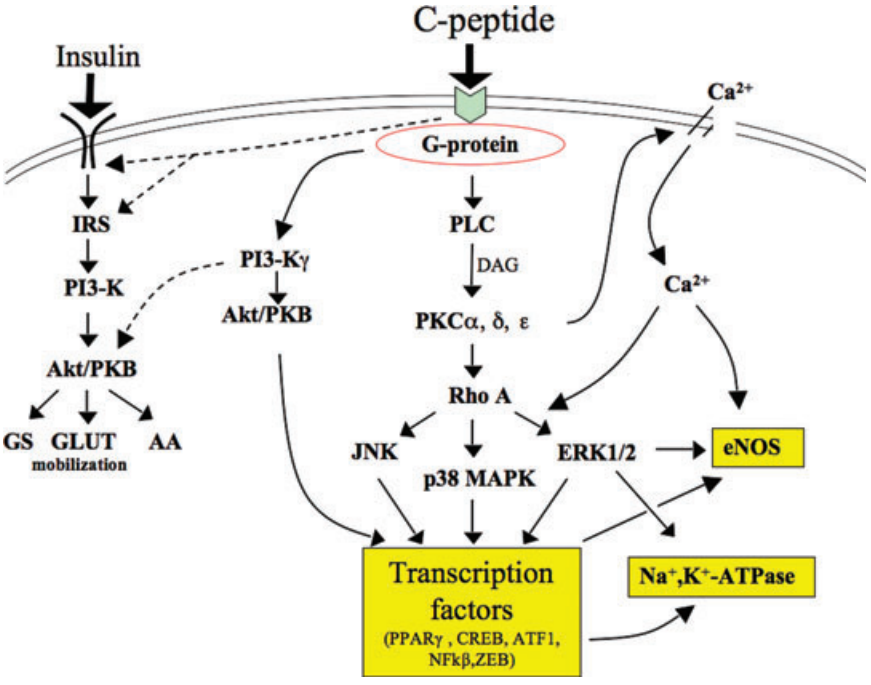

Fig. 13. Intracellular signaling by C-peptide C-peptide interaction with cell membranes results in activation of a pertussis toxin sensitive G-proteins. Subsequently, there is influx of Ca^{+2} into the cell and activation of endothelial nitric oxide synthase (eNOS), resulting in NO formation and local vasodilatation. Phospholipase C (PLC) and specific isomers of protein kinase C (PKC) are also activated as well as the mitogen-activated protein kinase (MAPK) complex. As a result, there is activation of Na^+,K^+–ATPase enzyme activity, but also DNA binding of several transcription factors, resulting in augmented eNOS mRNA formation and increased eNOS protein synthesis. Phosphoinositide 3-kinase (PI3-K) γ is also activated giving rise to peroxisome proliferator-activated receptor γ (PPAR-γ)-mediated transcriptional activity as well as augmented lymphocyte chemotaxis. In addition, there is evidence to indicate that C-peptide may interact synergistically on the insulin signaling pathway as indicated by dotted lines.

Na^+,K^+–ATPase

C-peptide exerts a direct stimulatory effect on Na^+,K^+–ATPase in renal tubular segments and tubular cells under *in vitro* conditions (99, 100). The effect is concentration-dependent at physiological concentrations. It is blockable by pertussis toxin and dependent on Ca^{2+}. *In vivo* studies of C-peptide's effects on sciatic nerve Na^+,K^+–ATPase activity confirm a stimulatory influence (77, 80). Moreover, red blood cell Na^+,K^+–ATPase activity is reduced in patients with T1D; the reduction is proportional to the decrease in C-peptide levels (118) and can be corrected by C-peptide administration (51) or after islet cell transplantation and restoration of endogenous C-peptide secretion (9). A secondary effect of the decreased red cell Na^+,K^+–ATPase activity is impaired deformability of the cells, which is corrected after exposure to C-peptide (119). Altogether, the evidence provides strong support for the existence of a direct relationship between C-peptide levels and Na^+,K^+–ATPase activity in renal and nerve tissue and in red blood cells under both *in vitro* an *in vivo* conditions.

C-peptide–insulin interaction

Insulin occurs in a hexameric form in the β-cells or in vials for insulin therapy but is biologically active only in monomeric form. Interactions between C-peptide and insulin oligomers have been studied using surface plasmon resonance and mass spectrometry. Unexpectedly, it was discovered that C-peptide influences the disaggregation of insulin by binding to insulin oligomers, with dissociation constants in the μm range (120). In accordance with this finding, mass spectrometry revealed that insulin hexamers in solution became non-detectable in the presence of C-peptide. Hence, C-peptide apparently binds to and causes disaggregation of hexameric insulin, increasing the availability of the biologically active monomeric insulin. Similarly, subcutaneous injection of an insulin and C-peptide mixture in T1D patients has been found to result in a more rapid appearance of insulin in plasma and more marked stimulation of glucose utilization compared to injection of insulin only (120). Thus, these results may present a molecular role for C-peptide in increasing the bioavailability of insulin by promoting the disaggregation of oligomeric insulin.

Structural conservation and cellular effects of C-peptide

The considerable structural variability among the C-peptides of 33 examined species – ranging from the Atlantic hagfish to the human (3) – was earlier considered an argument that C-peptide is unlikely to exhibit a defined biological activity. The mammalian C-peptide has typically 31 residues and includes four to five acidic residues. The central region varies with regard to its amino acid sequence and number of residues. Among the 20 known mammalian forms, nine residues localized to the N- and C-terminal segments show 90% or greater conservation. These are Glu1, Glu3, Gln6, Val7, Glu11, Leu12, Leu26, Glu27, and Gln31 (3). The conservation of the terminal residues Glu1 and Gln31 may be a consequence of the processing of proinsulin and the codon for Gln6 precedes an exon/intron junction. Glu3, Glu11, and especially Glu27 are known to be important for the cellular effects of C-peptide in as much as substitution of one or all of these with Ala results in substantial loss of biological activity as measured by MAPK phosphorylation (3, 104). Even though the species-dependent structural variability of C-peptide is considerable, C-peptide is not unique in this regard among bioactive peptides. Several peptide hormones show a similar degree of structural variability as C-peptide, e.g., parathyroid hormone and relaxin (121).

Summary and what next for C-peptide?

Undoubtedly, there is much more to learn about C-peptide. Identification of the mechanism whereby C-peptide interacts with cell membranes, delineation of its intracellular signaling pathways in different cell types, and further evaluation of its transcriptional effects will enhance our understanding of C-peptide bioactivity. On the clinical side further studies of longer duration (>6 months) will be required to document the robustness of its beneficial effects on the different types of long-term complications in order to define its possible role in the therapy of T1D. Nevertheless, despite the fact that our knowledge is still incomplete, there are several lines of unputdownable evidence in support of the notion that C-peptide is a bioactive peptide and that its replacement in T1D may be beneficial in the treatment of long-term complications. Even though the nature of the peptide's interaction with the cell membrane is only partially understood, its intracellular signaling characteristics and end effects including its action on eNOS, Na^+,K^+–ATPase, and several transcription factors are now well established for many cell systems and by different investigators. Results from studies in T1D patients and animal models demonstrate that C-peptide in replacement doses exerts beneficial effects on the early stage functional and structural abnormalities of both the kidneys and the peripheral nerves. The previous view that C-peptide is merely an inert by-product of insulin biosynthesis seems no longer tenable. Even a cautious evaluation of the available evidence presents the picture of a previously unrecognized bioactive peptide with

therapeutic potential in an area where no causal therapy is available today.

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Inflammation Research

ORIGINAL RESEARCH PAPER

C-peptide reduces pro-inflammatory cytokine secretion in LPS-stimulated U937 monocytes in condition of hyperglycemia

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Abstract
Objective We investigated C-peptide effects on inflammatory cytokine release and adhesion of monocytes exposed to high glucose and lipopolysaccharide (LPS) in vitro.
Materials and methods Monocytic cells (U-937) were cultured in the presence of 30 mmol/L glucose and stimulated with 0.5 ng/μL LPS in the presence or absence of C-peptide (1 μmol/L) for 24 h to induce inflammatory cytokine secretion. Adhesion of U-937 monocytes to human aortic endothelial cells (HAEC) was also studied in the presence or absence of C-peptide. Concentrations of IL-6, IL-8, macrophage inflammatory protein(MIP)-1α, and MIP-1β in supernatants from LPS-stimulated U-937 monocytes were assessed by Luminex. To gain insights into potential intracellular signaling pathways affected by C-peptide, we investigated nuclear translocation of nuclear factor(NF)-κB p65/p50 subunits by western blot in LPS-treated U-937 cells. The effect of C-peptide on LPS-induced phosphorylation of the cytoplasmic protein IκB-α was also investigated by immunoblotting.
Results Addition of C-peptide significantly reduced cytokine secretion from LPS-stimulated U-937 monocytes. Adhesion of U-937 cells to HAEC was also significantly reduced by C-peptide. These effects were accompanied by reduced NF-κB p65/p50 nuclear translocation and decreased phosphorylation of IκB-α.
Conclusions We conclude that, in conditions of hyperglycemia, C-peptide reduces monocytes activation via inhibition of the NF-κB pathway

Keywords Monocytes · Inflammation · Diabetes · C-peptide · NF-κB · Cytokines

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Introduction

Type 1 diabetes (T1D) is a well-established risk factor for vascular diseases. Patients with T1D exhibit an increased susceptibility to develop a wide range of vascular complications, including microangiopathy and accelerated atherosclerosis, which account for the majority of deaths and disability in diabetic patients [1, 2]. Vascular complications in diabetes arise from the combined action of exposure to abnormal blood glucose levels (i.e., hyperglycemia) and inflammatory responses generating oxidative stress and endothelial dysfunction. Inflammation and endothelial dysfunction are crucial processes in the pathophysiology of microvascular and other long-term complications associated with T1D [3].

Monocytes are pivotal cells in inflammatory responses as they serve as the principal reservoir of pro-inflammatory cytokines and are the first cells to be engaged in nonspecific immune responses, such as those triggered by environmental factors. Recent studies have reported the presence of increased monocytic activity, biomarkers of

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inflammation and oxidative stress in adults with established T1D [4, 5] as well as in children with recent-onset diabetes [6–8]. In these patients, monocytes release higher levels of pro-inflammatory cytokines, such as interleukin(IL)-1β, IL-6 and IL-8, in both the resting state as well as after lipopolysaccharide (LPS) stimulation as compared to non-diabetic subjects. Changes in monocyte function in T1D patients are associated with activation of the transcription factor nuclear factor κ-B (NF-κB) signal transduction pathway, and this activation is higher in patients with microvascular complications [9]. The direct consequences of monocyte activation in T1D are unknown, but theoretically could involve endothelial cell activation, oxidative stress, and increased monocyte adherence to small vessel walls, such as those of the heart and kidney. Overall, these findings support the idea that inflammatory responses underlie vascular compromise during T1D [9–11].

Strategies targeting NF-κB activation and inflammatory responses to inhibit several aspects of vascular dysfunction are emerging [12, 13]. In this context, C-peptide has been shown to antagonize endotoxin-induced inflammatory responses in mice [14] as well as down-regulating inflammatory cytokine secretion and adhesion molecule expression in endothelial cells exposed to a variety of inflammatory insults [15–17]. Animal studies and clinical phase I/II trials in patients with T1D who take C-peptide as replacement therapy provide direct evidence of a protective effect of C-peptide against diabetes-induced complications of the kidneys, the peripheral nerves, and CNS function [18–20]. In addition, it has been shown that T1D patients with residual beta cell function or receiving whole pancreas or allogeneic islet transplantation do not develop long-term complications, a result that could be attributed to a positive effect of C-peptide [21–25]. These results identify C-peptide as a compelling novel therapeutic candidate in T1D.

In addition to endothelial cells, circulating monocytes might represent an additional cellular target for the anti-inflammatory effect of C-peptide. Whether C-peptide displays any direct biological effect(s) on human monocytes under conditions of high glucose has not been investigated to date. This study was specifically aimed at investigating the possible anti-inflammatory effect(s) of C-peptide on human monocyte biological functions in conditions of hyperglycemia, a common condition in T1D. To induce cytokine release, U-937 monocytes were stimulated for 24 h with LPS in the presence or absence of full-length native human C-peptide. Adhesion of U-937 monocytes to a monolayer of HAEC was also studied in the presence or absence of C-peptide. Finally, involvement of the NF-κB pathway in the intracellular signaling activity underlying C-peptide effects on U-937 monocytes was investigated. The results of this study demonstrate that monocytes represent an additional cellular target for C-peptide anti-inflammatory activity that may have an impact on the prevention of endothelial dysfunction in T1D.

Materials and methods

Cell culture of the human monocytic cell line U-937

Human monocytic U-937 cells were purchased from the American Type Culture Collection (Rockville, MD) and grown in complete RPMI-1640 (Lonza, Walkersville, MD, USA) supplemented with 10% FBS, ***100 µl/mL streptomycin, 100 IU/mL penicillin, 250 ng/mL fungizone, 1 mmol/L Sodium Pyruvate and 10 mmol/L HEPES (all from Gibco Invitrogen, Carlsbad, CA, USA) in ***T75 cm^2 flasks (Corning, NY, USA) at 37°C, 5% of CO_2.

LPS-stimulation of U-937 monocytes for cytokine secretion study

U-937 cells (50,000 cells/well/mL) were seeded in 24-well plates in presence of RPMI-1640 supplemented with 30 mmol/L glucose and stimulated with 0.5 ng/µL of LPS from *E. coli* (E25:B55) in the presence or absence of 1 µmol/L C-peptide (Sigma Chemical, St. Louis, MO, USA) for 24 h at 37°C, 5% of CO_2. A randomized version of the full-length C-peptide containing the same amino acid residues but randomly ordered (scrambled C-peptide; Sigma Genosys), was used as control at the same concentration of the C-peptide [26]. Supernatant was collected in 1.5-mL cryovials and stored at −20°C until tested for cytokine concentration. Luminex multiplex assays were used to assess levels of IL-6, IL-8, macrophage inflammatory protein (MIP)-1α and MIP-1β (Millipore Human Cytokine kit; Millipore, Billerica, MA, USA) in the culture supernatants of LPS-stimulated U-937 cells following the manufacturer's instructions. A minimum of 4 independent experiments were performed. Each condition was tested in duplicate.

Adhesion of U-937 monocytes to Human Aortic Endothelial Cells (HAEC)

HAEC were purchased from Lonza and maintained in EBM-2 media at 37°C, 5% CO_2 as previously described [17]. HAEC (200,000 cells/well) were then seeded on 48-well plates and grown until they reached 90% confluency. U-937 monocytes were exposed to low (5.6 mmol/L), or intermediate (11 mmol/L), or high glucose (30 mmol/L) medium, in the presence or absence of C-peptide (2 µmol/L) for 4 h at 37°C, 5% CO_2. As a positive control to induce adhesion, U-937 monocytes were stimulated with IL-1β (1 ng/mL; Sigma Chemical) for 4 h at 37°C, 5% CO_2. After the allotted time, U-937 cells were added on top of the HAEC for 1 h at room

temperature on a rocking plate. Non-adherent U-937 cells were removed by washing, and adherent cells fixed in 1.25% glutaraldehyde. The number of adherent U-937 monocytes was evaluated by counting five random 40× fields per well [17, 27]. At least three experiments were performed. Within each experiment, each condition was tested in triplicate.

NF-κB p65/p50 analysis assays

U-937 cells were cultured in 75 cm^2 culture flasks (Corning) in complete RPMI-1640 with 30 mmol/L glucose and exposed to LPS (0.5 ng/μL) in the presence or absence of C-peptide (1 μmol/L) for 24 h. Cells were harvested and pretreated with 25 μl of protease inhibitor cocktail (Pierce, Rockford, IL, USA). Nuclear and cytoplasmic fractions were separated using NE-PER® Nuclear and Cytoplasmic extraction kit (Pierce). Protein content of the extract was measured using a bicinchoninc acid assay kit (Pierce). Aliquots of protein extracts (20 μg) were resolved on 4–20% SDS-PAGE. Proteins were blotted onto PVDF membranes, which were subjected to immunoblot assay using anti-NF-κB p65 antibody (1:1,000 dilution; Santa Cruz Biotechnology) as previously described [28]. Densitometry analysis of the bands was performed with UN-SCAN-IT gel software (Silk Scientific). Activation of the NF-κB p50 subunit was detected on 3 μg of nuclear protein extracts using an EZ-Detect™ Transcription Factor Kit (Pierce). For each set of data, a minimum of three experiments were performed. Data were averaged and expressed as means ± SD.

Immunoprecipitation of IκB-α and phosphorylated-IκB-α

Cytoplasmic extracts (50 μg) were incubated overnight at 4°C with anti-IκB-α IgG (1:100). The samples were then treated with 10 μl of protein A agarose beads (Sigma) for 2 h at 4°C after which the samples were centrifuged and washed 5 times in PBS (Gibco). The beads were boiled in SDS-PAGE sample treatment buffer and electrophoresed on a 4–20% SDS-PAG as described above. The PVDF membranes were then blotted and incubated with either monoclonal P-IκB-α or IκB-α antibodies (both from Santa Cruz Biotechnology; 1:1,000) followed by incubation with an anti-rabbit peroxidase secondary antibody (1:10,000; Jackson Laboratories, Bar Harbor, ME, USA).

Statistical analysis

Two-tailed paired t test was used to assess differences between the different conditions tested by using GraphPad Prism 4 program (GraphPad Software, San Diego, CA, USA). Values of $p < 0.05$ were considered to be statistically significant.

Results

C-peptide reduces pro-inflammatory cytokine secretion by LPS-treated U-937 monocytes

As expected, exposure of U-937 cells to high glucose and LPS for 24 h significantly stimulated secretion of several pro-inflammatory cytokines, such as IL-6, IL-8, MIP-1α, and MIP-1β as compared to cells in high glucose medium alone (Fig. 1). Addition of C-peptide (1 μmol/L) to LPS-treated U-937 monocytes significantly reduced secretion of IL-6, IL-8, MIP-1α and MIP-1β (Fig. 1). Addition of scrambled C-peptide (1 μmol/L) did not significantly affect LPS-induced cytokine secretion after 24 h incubation (Fig. 1).

C-peptide inhibits high glucose-induced adhesion of U-937 cells to HAEC

We assessed adherence of U-937 monocytes to HAEC after exposure of monocytes to C-peptide and glucose at various concentrations. When C-peptide was added to 30 mmol/L glucose, there was a significantly decreased number of adherent monocytes as compared to high glucose alone ($p < 0.05$) (Fig. 2a). As a positive control, IL-1β was used to stimulate adherence of U-937 monocytes to HAEC ($p < 0.01$ vs. high glucose). In low (5.6 mmol/L) and intermediate (11 mmol/L) glucose conditions, C-peptide did not show significant effects on U-937 monocyte adherence compared to medium without C-peptide. In Fig. 2b are shown representative images of U-937 monocyte adhesion under the different conditions tested.

The NF-κB pathway is affected by C-peptide in LPS-stimulated U937 monocytes

The signal transduction pathway leading to mRNA synthesis of adhesion molecules and cytokines involves activation of NF-κB. To determine whether C-peptide affected high glucose-induced NF-κB nuclear translocation in LPS-stimulated U-937 monocytes, immunoblot analysis and NF-κB-specific ELISAs were performed with nuclear extracts from LPS-stimulated U-937 cells. As shown in Fig. 3a, exposure of U-937 cells to LPS for 24 h induced an increase in NF-κB nuclear translocation in comparison to cells without LPS-stimulation. Densitometry analysis of the western blots demonstrated a twofold increase in NF-κB p65 activation after LPS-stimulation as compared to incubation with 30 mmol/L glucose alone ($p < 0.05$) (Fig. 3a, b). Addition of C-peptide (1 μmol/L) significantly decreased NF-κB p65 nuclear translocation as compared to stimulation with LPS only ($p < 0.05$) (Fig. 3a, b).

Addition of C-peptide to LPS-treated U937 monocytes also reduced NF-κB p50 subunit nuclear translocation as

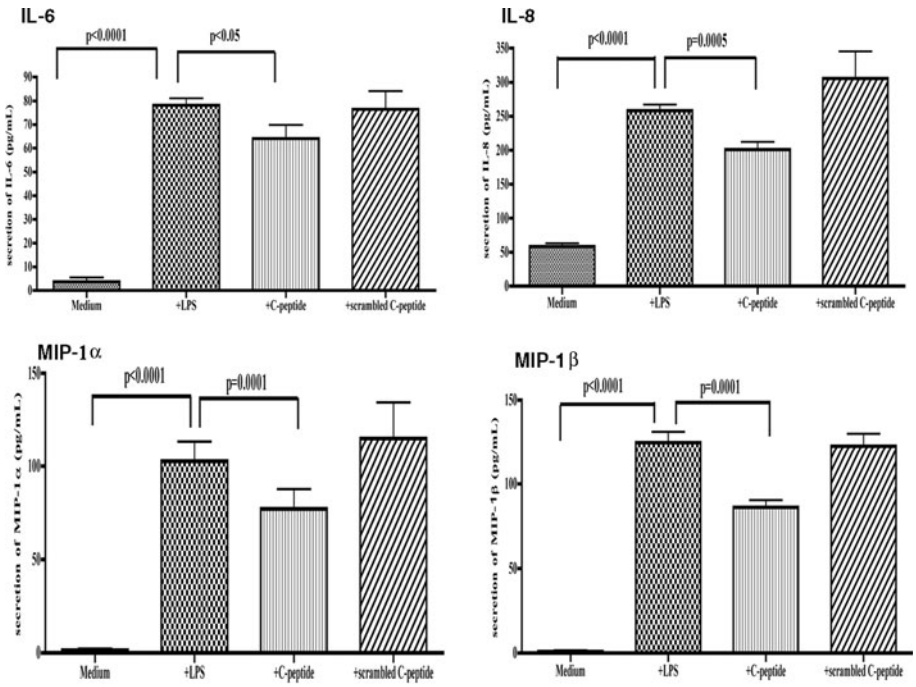

Fig. 1 C-peptide reduces LPS-stimulated secretion of *IL-6*, *IL-8*, *MIP-1α*, and *MIP-1β* in U937 monocytes. U-937 cells were cultured with 30 mmol/L glucose and stimulated with 0.5 ng/μL of Lipopolysaccharide (LPS) in the presence or absence of 1 μmol/L C-peptide for 24 h. Scrambled C-peptide (1 μmol/L) was used as a control. Luminex multiplex assays were used to assess levels of IL-6, IL-8, MIP-1α and MIP-1β in the culture supernatants of LPS-stimulated U937 cells. *Bar graphs* demonstrate mean ± SD of cytokine secretion (pg/mL). Cells exposed to LPS produced more cytokines than cells exposed to high glucose alone ($p < 0.001$). Addition of C-peptide to the LPS-treated U-937 monocytes reduced secretion of IL-6, IL-8, MIP-1α and MIP-1β ($p < 0.05$, $p = 0.0005$, $p = 0.0001$, $p = 0.0001$, respectively). Addition of scrambled C-peptide to the culture medium did not significantly affect LPS-induced cytokine secretion. A minimum of 4 independent experiments were performed. Each condition was tested in duplicate

compared to LPS-treatment alone, as detected by ELISA ($p = 0.0042$) (Fig. 4).

The mechanism underlying NF-κB nuclear translocation from the cytoplasm to the nucleus is based on the phosphorylation of IκB-α. We therefore investigated the effects of C-peptide on LPS-induced phosphorylation of IκB-α by western blotting on cytoplasmic extracts from U-937 cells (Fig. 5). As expected, an increase in the level of phosphorylated-IκB-α was observed in the cytoplasmic extracts of LPS-stimulated U937 monocytes after 24 h treatment as compared to U-937 cells cultured in 30 mmol/L glucose alone (Fig. 5). Addition of 1 μmol/L C-peptide caused a decrease in the level of phosphorylated-IκB-α as compared to cells exposed to LPS in the absence of C-peptide (Fig. 5).

Discussion

T1D is a well-known risk factor for both micro- and macrovascular disease with inflammation and endothelial dysfunction being major contributors to this risk. Findings indicate that a generalized inflammatory response is already present in the very early stages of diabetes [6, 8]. Furthermore, T1D patients with microvascular complications exhibit increased production of inflammatory biomarkers, such as pro-inflammatory cytokines, over and above the levels detected in T1D patients without microvascular complications and healthy control subjects [9–11, 29]. A number of the inflammatory changes in T1D are detected at the level of monocyte cell. For example, up-regulation of the adhesion molecule CD11b (Mac-1) has been demonstrated [8] in addition to aberrant constitutive and LPS-stimulated expression of cyclooxygenase (COX)-2, a defect that may predispose to a chronic inflammatory response in T1D [30, 31]. The vascular endothelium represents a likely target of this inflammatory response detected in patients with T1D.

In this study, we demonstrate that activated U-937 monocytic cells represent an additional target for the anti-inflammatory activity of C-peptide in diabetes. C-peptide significantly reduces high glucose-stimulated secretion of IL-6, IL-8, MIP-1α, and MIP-1β from LPS-treated U-937 monocytes. Additionally, when C-peptide was added to 30 mmol/L glucose, there was a decreased number of adherent monocytes to HAEC as compared to high glucose alone. In low (5.6 mmol/L) and intermediate (11 mmol/L) glucose conditions, C-peptide did not show any significant effects on the adherence of the treated U-937 monocyte to

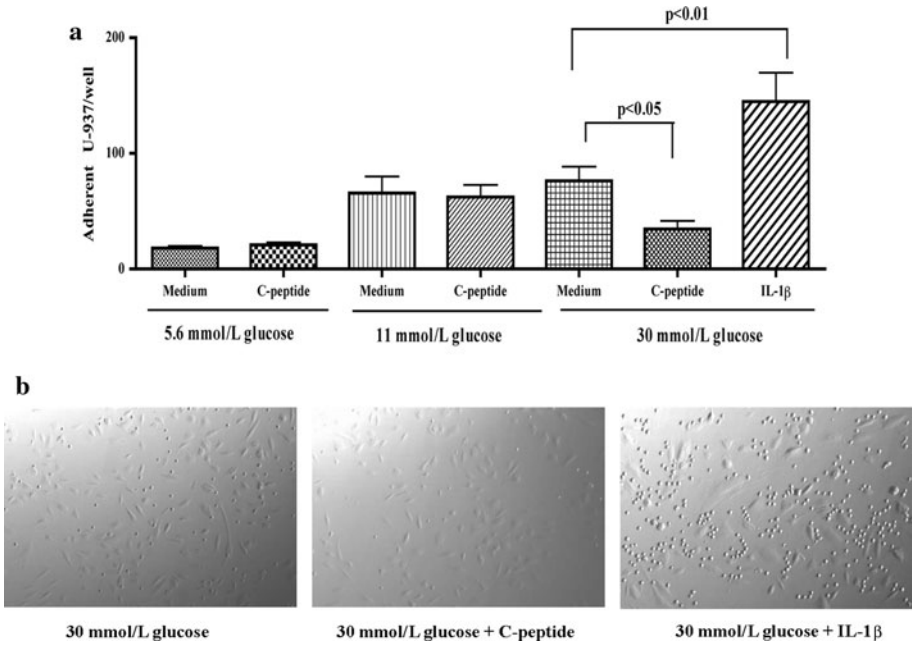

Fig. 2 C-peptide reduces adhesion of U937 monocytes exposed to high glucose to HAEC. HAEC (200,000 cells/well) were seeded in 48-well plates and grown at confluency. U-937 monocytes were exposed to either low (5.6 mmol/L), or intermediate (11 mmol/L), or high glucose (30 mmol/L) medium, in the presence or absence of C-peptide (2 μmol/L) for 4 h at 37°C, 5% CO_2. After the allotted time, U-937 cells were added on top of the HAEC for 1 h on a rocking plate and after washing out the non-adherent cells, cells were fixed in 1.25% glutaraldehyde. **a** C-peptide significantly reduces the number of high glucose-treated U937 monocytes adherent on HAEC as compared to condition without C-peptide ($p < 0.05$). IL-1β (1 ng/mL) was used to stimulate adherence of U-937 monocytes to HAEC ($p < 0.01$ vs. high glucose). The number of adherent U-937/well expressed as mean ± SD of at least three different experiments, in which each condition was tested in triplicate, are shown. **b** Photographic images of U-937 monocyte adhesion under the various conditions tested

HAEC, thus suggesting that C-peptide exerts its most beneficial effects on the activated monocytes in conditions of insult from extreme hyperglycemia. Consistently, hyperglycemia, a major clinical sign in T1D, has been recognized to play a crucial role in the development of endothelial dysfunction leading to vascular complications in T1D [2].

Endothelial cells, vascular smooth muscle cells, and monocytes are three cellular components, each of which plays a lead role in vascular dysfunction. Our current findings extend previous work from our group demonstrating an anti-inflammatory activity of C-peptide on both endothelial cells and vascular smooth muscle cells exposed to high glucose [17, 32]. In particular, we found that C-peptide reduced high glucose-induced up-regulation of the endothelial adhesion molecule vascular cell adhesion molecule(VCAM)-1, and decreased pro-inflammatory cytokine secretion from the same cells [17]. Additionally, we found that C-peptide decreased high glucose-induced vascular smooth muscle cell proliferation by affecting the same intracellular pathway [32]. Other groups have published on the anti-inflammatory effects of C-peptide on the inflamed endothelium in different models of vascular injury [14, 16]. More recently, injections of C-peptide in vivo to LPS-treated mice, an animal model of endotoxic shock, drastically improved survival rate of the animals compared to the vehicle-treated mice, an effect associated with reduced plasma levels of the inflammatory cytokines tumor necrosis factor (TNF)-α and monocyte chemoattractant protein (MCP)-1 and to a decreased overall inflammatory response in the lung [14], an organ severely damaged in endotoxic shock. Overall, these findings emphasize the concept that C-peptide possesses anti-inflammatory activity on several different cellular targets.

The signaling pathway that activates the transcription factor NF-κB regulates the transcription of a number of genes that are involved in inflammation and proliferation. In the unstimulated state, NF-κB exists as a heterodimer in the cytoplasm composed of p50 and p65 subunits bound to IκB [33]. In response to different stimuli, IκB is phosphorylated and degraded causing a release of the p50/p65 subunits, which subsequently translocates to the nucleus and initiates the transcription of different genes involved in the inflammatory response, such as those encoding for pro-inflammatory cytokines, cell surface adhesion molecules, and chemokines [33]. The NF-κB pathway has been implicated in vascular disease, and the activated p65 subunit has been found expressed in the fibrotic thickened

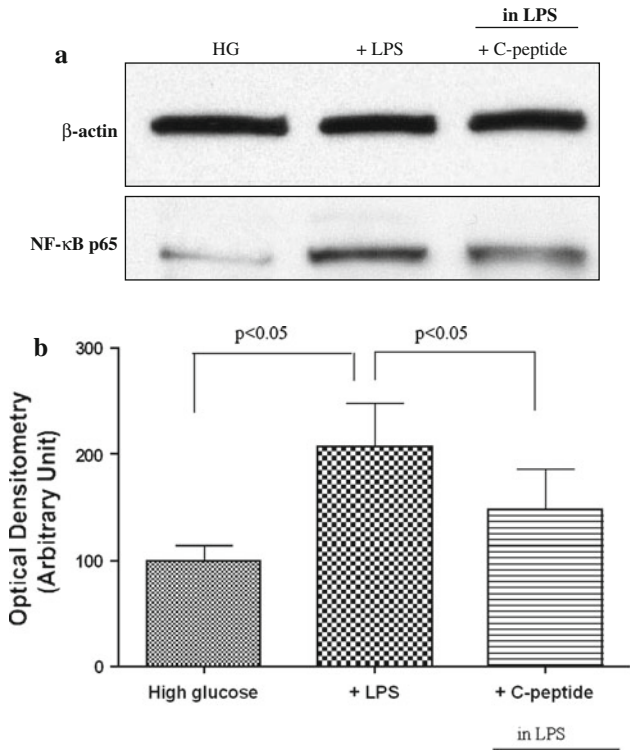

Fig. 3 C-peptides reduces nuclear translocation of the NF-kB p65 subunit in LPS-stimulated U-937 cells. U-937 cells were cultured in 75-cm^2 culture flasks in complete RPMI-1640 with 30 mmol/L glucose (*HG*) and exposed to *LPS* (0.5 ng/μL) in the presence or absence of *C-peptide* (1 μmol/L) for 24 h. **a** Nuclear extracts were subjected to western blotting to detect p65 subunit of NF-κB. **b** The densitometric quantification (as arbitrary units; AU) of the bands detected in 3 different experiments. In cells exposed to LPS, there was a twofold increase in NF-κB p65 nuclear translocation compared with cells in HG alone ($p < 0.05$). A significant decrease in p65 nuclear translocation was detected in the presence of 1 μmol/L C-peptide ($p < 0.05$ vs. LPS). Results are expressed as mean ± SD ($n = 3$ experiments)

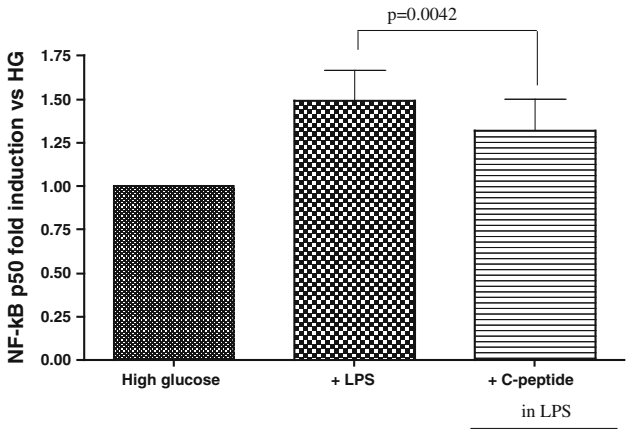

Fig. 4 C-peptides diminishes nuclear translocation of NF-κB p50 subunit in LPS-stimulated U937 cells. Detection of the NF-κB p50 subunit by ELISA in nuclear extracts from U-937 monocytes exposed to *high glucose* (30 mmol/L), *LPS* (0.5 ng/μL), or *LPS* (0.5 ng/μL) + *C-peptide* (1 μmol/L) for 24 h. Results are expressed as fold induction of NF-κB p50 expression levels in respect to 30 mmol/L glucose. In cells exposed to LPS, there was a 1.5 increase in NF-κB p50 expression level compared to cells in HG alone. A decrease in NF-κB p50 expression level was observed in the presence of C-peptide ($p = 0.0042$ vs. LPS condition)

intima-media and atheromatous areas of the atherosclerotic lesion, macrophages, endothelial cells, and smooth muscle cells [34]. In this study, we demonstrate that, similar to endothelial and vascular smooth muscle cells [17, 32], in monocytes C-peptide also achieves its anti-inflammatory activity by intracellular mechanisms leading to a reduction in NF-κB activation. Western blot and ELISA analyses of nuclear extracts from LPS-treated U-937 cells exposed to high glucose showed a decreased nuclear translocation of NF-κB p50 and p65 subunits through a decreased phosphorylation of the cytoplasmic inhibitory protein IκB. Although the exact mechanism by which C-peptide affects phosphorylation of IκB is not known, one possible mechanism envisages that C-peptide turns off the cascade of phosphorylation events ultimately leading to the activation of kinase proteins involved in the phosphorylation of the inhibitor protein IκB. By shutting down the phosphorylation cascade, C-peptide might interfere with the dissociation of IκB:NF-κB complex in the cytoplasm, limiting the migration of p65/p50 subunits to the nucleus, thus reducing the activation of genes involved in inflammatory processes. The phosphorylation cascade is carried through recruitment of several kinase proteins such as IκB, IKKα and IKKβ, and is initiated at the level of the plasma membrane after the binding of a ligand to a membrane receptor (e.g., Toll-like receptor). At which level of the phosphorylation cascade C-peptide might act is unknown. Another NF-κB-dependent upstream event that needs to be elucidated as a potential target for C-peptide effect is reactive oxygen species (ROS) generation. ROS are powerful cellular activators of the NF-kB pathway. C-peptide might reduce NF-κB activation by decreasing ROS generation in U-937 monocytes. In this case, one cellular target for C-peptide effect on decreasing ROS generation is the NAD(P)H oxidase enzyme, which is one pathway regulating ROS production in U-937 monocytes [35, 36].

In conclusion, our findings support the hypothesis that circulating monocytes represent an additional cellular target for the anti-inflammatory effect of C-peptide in conditions of hyperglycemia, a common condition in T1D. Furthermore, the anti-inflammatory activity of C-peptide is likely due to suppression of NF-κB activation. These findings underscore the role C-peptide may play in the prevention of endothelial dysfunction in T1D which leads to micro- and macrovascular disease, both major causes of morbidity and mortality in T1D. Insulin alone, the mainstay of treatment for T1D, has been shown to exert anti-inflammatory properties in a number of settings, including

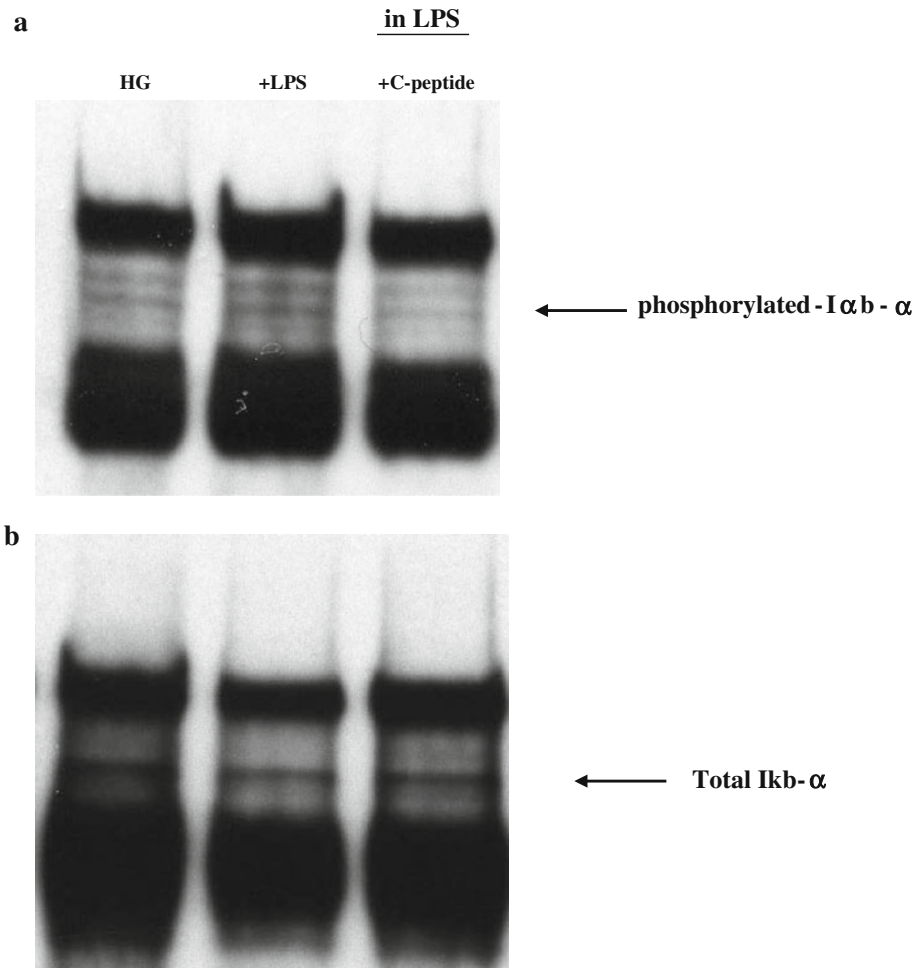

Fig. 5 Inhibitory effect of C-peptide on phosphorylation of IκB-α protein in LPS-stimulated U-937 monocytes. Cytoplasmic extracts (50 μg) from U-937 cells treated with high glucose (*HG*) (30 mmol/L), *LPS* (0.5 ng/μL), or *LPS* (0.5 ng/μL) + *C-peptide* (1 μmol/L) were incubated overnight at 4°C with anti-IκBα IgG (1:100). The samples were then treated with 10 μl of protein A agarose beads for 2 h at 4°C after which the samples were centrifuged and washed in PBS. The beads were boiled in SDS-PAGE sample treatment buffer and electrophoresed on a 4–20% SDS-PAG. The gel was blotted and incubated with either monoclonal P-IκBα or IκBα antibodies (both from Santa Cruz Biotechnology; 1:1,000). **a** An increase in the level of phosphorylated-IκB-α was observed in the cytoplasmic extracts of LPS-stimulated U-937 monocytes after 24 h treatment compared to U-937 cells cultured in 30 mmol/L glucose alone. Addition of C-peptide caused a decrease in the level of phosphorylated-IκBα as compared to cells exposed to LPS in the absence of C-peptide. **b** Total IκB-α levels did not change upon treatment conditions

settings designed to simulate T1D [37–39]. It is clear that c-peptide also possesses anti-inflammatory properties.

The beneficial effects of combined insulin and c-peptide therapy (compared to insulin alone) have been demonstrated in patients with T1D with respect to sensory nerve function [18], nephropathy and neuropathy [19]. The effect of insulin and C-peptide co-treatment has also been investigated in vivo and in vitro, showing reduced hippocampal apoptosis in T1D rats [20] and increasing human cavernosal smooth muscle cell relaxation through increased generation of eNOS and iNOS [40], respectively. A recent study carried out by Mughal et al. [41] investigated the interplay between human insulin and proinsulin C-peptide in an in vitro model of intimal hyperplasia in saphenous vein bypass grafts. The study showed that co-treatment of human insulin and C-peptide limited the neointima formation in cultured smooth muscle cells, indicating that a co-administration of insulin and C-peptide may improve saphenous vein bypass potency rates in T1D patients. Human neuroblastoma SH-SY5Y cells [42] and opossum kidney proximal tubular cells [43] represent additional in vitro models that have responded positively to insulin and C-peptide co-treatment. Given the beneficial properties of C-peptide in a number of experimental settings together with clinical studies demonstrating the added benefit of C-peptide to therapy with insulin, one may speculate dual hormone replacement therapy with both insulin and C-peptide in patients with T1D may be

warranted in the future. Further studies need to be performed to delineate C-peptide's exact biological role.

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